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Recombinant DNA: local or federal regulation?

AFTER raging through college campuses and state and local government offices throughout the United States, the debate over the risks and benefits associated with recombinant DNA experiments has now reached a critical juncture in the US Congress. In the next few weeks, committees in both the House of Representatives and the Senate are expected to approve their own versions of legislation to regulate recombinant DNA experiments. Although substantial differences of opinion between the House and Senate committees are emerging on key aspects of the legislation, one point in particular has become the focus of a major lobbying effort by members of the scientific community: the extent to which federal regulations governing recombinant DNA activities should over-ride controls adopted by state and local governments.

Some important points of principle are at stake here, and it is no surprise that the matter has become the centre of a controversy. The dilemma facing Congress is this. On the one hand, it would make no sense to have a patchwork of state and local controls on recombinant DNA research so that experiments were regulated more tightly in one city than in another. Genetically modified micro-organisms, after all, would not respect political boundaries, and a uniform set of national (or, preferably, international) controls would therefore make scientific sense. But on the other hand, the debates which have resulted in state and local governments adopting their own regulations have witnessed considerable public participation in scientific decision-making and the forging of new links between universities and their surrounding communities—ideals often given lip service but seldom put into practice. If the federal government takes away the right of local governments to set their own recombinant DNA regulations, it would risk stifling such developments. Moreover, there is some concern about the political implications of usurping a community's right to decide whether potentially hazardous activities should be permitted in its midst.

Needless to say, the scientific community has come down heavily on the side of federal pre-emption. Indeed, some scientists have publicly stated that they would support federal legislation only if it establishes uniform national regulations. The most prominent statement of such a position came late in April, when the National Academy of Sciences approved a resolution urging Congress to write a provision into the recombinant DNA bill specifically stating that federal controls would over-ride all local regulations. The Academy's resolution, it is

worth noting, was endorsed by Robert L. Sinsheimer, of Caltech, a leading critic of current guidelines governing recombinant DNA experiments.

But there has also been weighty testimony from a few scientists and from state and local officials, who have argued that the intervention of local governments in the recombinant DNA debate so far has been beneficial and there is no reason to expect that arbitrary or excessively strict local control will be adopted in the future. Every state and local government which has acted so far has endorsed the NIH guidelines, added a few minor additional precautions, and adopted procedures to enforce them. Even in Cambridge, where town-gown relationships had been notoriously bad, the city council approved recommendations, put forward by a citizens' committee, allowing recombinant DNA experiments to go ahead at Harvard and MIT essentially under the NIH guidelines. The report of the citizens' committee, in fact, provides an excellent example of how a group of lay people can participate effectively in scientific decision-making.

Is it therefore possible to establish uniform national controls without shutting off legitimate and healthy local inquiries? Possibly. Although some key legislators are hanging firmly to the notion that states should be given free licence to do as they please, a consensus seems to be developing in support of a scheme which would allow local changes to national regulations only under special circumstances. Perhaps more important, the scheme would provide local communities with an opportunity to have some input into national regulations.

It would work like this. The Secretary of Health, Education and Welfare (or whoever administers the national regulations) would have to approve local changes to the national rules. Approval would be given only if the local regulations are more strict than the national rules and if the local government can demonstrate that there are special local circumstances or that they are necessary to protect public health. Opportunity would be given for public hearings on the proposed changes. In practice, if a local government can make a good case, it would be likely to trigger changes in the national rules.

Although the arrangements would limit the powers of local government in the recombinant DNA debate, it would give them more authority than they have in areas such as radiation standards and the regulation of food additives. In short it represents a reasonable compromise between total federal pre-emption and the possibility of widely varying local controls. □



Under a cloud of committees

From Sydney, Peter Pockley updates some recent developments in Australian science policy

THE science-and-politics scene in Australia in the first half of 1977 has been remarkable only for much running on the spot with accompanying huff and puff. The symptoms are characteristic of SIC, the Syndrome of Inquiry and Committee, which, whether caught adventitiously or implanted deliberately, is manifest through a distinct brake on progress.

Those looking for a plot behind every move of the present conservative government might adduce SIC as evidence of a concerted move to downgrade the national science effort, the end result being disastrous attrition of the research dollar through galloping inflation before any substantive action occurs. But, on unravelling who is doing (or not doing) what among the dozen or so federal government departments with a major stake in science and technology, it is not surprising to find little more than a classic case of uncoordination.

One positive aspect of SIC is the appearance it gives of greater sympathy within the Liberal/Country Party government towards science, notably its applied aspects, than obtained within its Labor predecessor, in spite of the fact that under Labor science policy discussions were more public than before. After 18 months in office, though, the Fraser government's interest is still expressed only by SIC and the maintenance, for the time being, of the status quo. Everyone now expects the funds for science to remain very tight while high inflation (11–15%, depending on time-scale and assumptions) and high unemployment (5.5%, the highest since the Depression years) dominate the economic climate. Nonetheless, there are some government inquiries and committees which have the potential, at least, to affect constructively the course of events.

Breathing space

Late last year, a three-man Committee of Inquiry was established to look thoroughly into Australia's research colossus, the Commonwealth Scientific and Industrial Research Organisation (CSIRO). The full-time Chairman, seconded for the purpose from the Australian National University, is Professor Arthur Birch, an organic chemist noted for his contribution to the contraceptive pill. The other two members of the committee are a financier and an industrialist. Their appointments caused some tremors that the

government was intending, through the inquiry, to commercialise CSIRO by demanding short-term financial benefits from its research.

The final clause of the comprehensive terms of reference for the inquiry required the committee to "examine, report, and make recommendations on the extent to which and the means by which programs of the Organisation could attract revenue both to support the conduct of ongoing or intended research and also in return for results achieved in research".

The inquiry, though, ranges far wider than this. While it was not intended as a mere cost-benefit study of the operations of CSIRO's 37 divisions and 100-plus operations and \$140 million-plus budget, the pressure was on the members to come up with an interim report early in the year. The urgency was felt because of the imminence of the retirement in March of CSIRO's Chairman, Sir Robert Price (formerly Dr Jerry R. Price). The inquiry was asked to answer the question of what kind of person is needed to head the organisation, but an organisation that before long would presumably have some new terms of reference from the government as a result of the inquiry's own recommendations, which would have been either tentative or unframed at that time.

The government solved the inquiry's dilemma by appointing, for 12 months only, a new chairman in Mr Victor Burgmann, a long-standing member of CSIRO's Executive. The inquiry now has some breathing space for its major tasks, on which it was originally expected to report to the Prime Minister by the end of July.

Professor Birch says the inquiry is "not trying to put a few band-aids on CSIRO, but is taking a hard look at the whole set-up—where it fits in between universities and industry, and between Commonwealth and State departments and instrumentalities. While the benefits from cost-benefit analysis of CSIRO's research will be very hard to measure, we are making strenuous efforts to gain a good idea of them."

The Prime Minister showed his close interest in the inquiry by issuing the commission himself and, although CSIRO is responsible under its Act to the Minister for Science, Senator James Webster, the report will go to Mr Fraser.

Professor Birch says he has no political connections and he hopes the in-

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Malcolm Fraser, Prime Minister

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James Webster, Minister for Science

quiry's recommendations ("not gospel from heaven") will be acceptable to both sides of Parliament, for he sees CSIRO's operations as "lying outside the range of normal party politics". Nevertheless, given the continuing silence of the Labor Party on matters scientific, it is likely that Mr Fraser will be able to remould CSIRO's terms of reference to his own liking.

Going autonomous

The longest running stage show in Australian science—the on-again, off-again saga of a science council in various costumes—has at last been concluded with an announcement that the independence, permanence and stability of a statutory authority would be granted to ASTEC, the Australian Science and Technology Council. ASTEC proper, however, still has to await the presentation and passage of legislation through Parliament.

At least ten years of lobbying of successive Prime Ministers and Ministers for Science finally bore fruit when Mr Fraser announced on 19 April that he had accepted, in large measure, the recommendation of the Interim

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Uranium uncertainty

THOSE on either side of the nuclear power debate who were hoping for a clear 'stop' or 'go' for uranium mining in Australia and uranium export have been left disappointed by the second report of the Ranger Uranium Inquiry. The report, prepared by Mr Justice Fox of the Australian Capital Territory Supreme Court and his two fellow commissioners, was published on 25 May by the Australian Government Publishing Service.

It included several surprises for the speculators, as indicated by the wild fluctuations in the share market for uranium mines immediately after the report's much publicised release. On the specific problems of mining uranium in the Northern Territory, the report, like its predecessor handed down in October 1976 which dealt more with the global issues of safeguards and controls, has left the ball firmly in the government's court.

- There is something in it for the uranium lobby. The Ranger mine is given a cautious go-ahead, though under much stricter and very specific (hence more expensive) conditions regarding environmental control.

- There is something in it for the environmentalists. It is recommended that "as proposed, and in the land setting which was proposed, the Ranger project not be allowed to proceed". Further and larger mines nearby in the uranium-rich Alligator Rivers province, the development of which was also halted pending the Ranger report, are recommended

"not to proceed, at least for the time being". The main mines delayed by the proposed sequential development are the Noranda and Jabiluka mines.

- There is even more in it for the aborigines whose claims to land in the area cover most of the uranium province. The report is strongest of all on this matter, recommending a trebling of the Kakadu National Park to cover the entire Alligator Rivers region, yet warning that the greatest threat to the environment and particularly to the welfare, well-being and culture of the Aboriginal people, might prove to be the large white population which the mining ventures might bring.

The Prime Minister, Mr Fraser, left for overseas on the day after the report's release. He has signalled that his discussions with President Carter and other leaders have uranium policy high on the agendas, especially since Mr Carter's staff have been saying, somewhat paradoxically for the layman, that the new US policy for containment of nuclear proliferation relies heavily on the export of Australian uranium.

While the Australian government has made no formal decision and has announced that a full debate will not occur in Parliament until the Budget session in August, few people doubt that mining will be allowed to proceed. The only real question is which mines will start and when. Meanwhile, the public relations and political lobbying of the pro- and anti-nuclear

forces will intensify, each side claiming to summarise accurately in a few words that the highly complex and voluminous Fox report supports their side.

On a wider front, however, Australia has joined the political bandwagon that is energy more by reason of following the leader (that is the USA and Europe) than of any urgency felt imperative locally. Australia is blessed with vast black and brown coal deposits, there are some modest oil wells and reasonable gas fields, and there has been a government policy of keeping down the prices of petroleum products. Despite the need to import increasing quantities of crude oil, few Australians have felt the effect of a genuine scarcity of energy. Politically, energy has not yet equated with votes.

Nonetheless, the Deputy Prime Minister, Mr Doug Anthony, has seized on the energy question as his rightful responsibility under his role as Minister for National Resources. His views on the matter are now accorded respectful space in the press, a good start to making energy a real issue. But, while there is much talk of "moving towards an energy policy", nobody is really saying what it is likely to be.

Mr Anthony has shown willingness to listen to expert opinion, and has set up an 18-person National Energy Advisory Committee chaired by top metallurgist, Dr Howard Worner. It is charged with advising on Australia's energy reserves, supply and demand, future costs, economy of use, research and technology.

In a welcome move, Mr Anthony has decided to release the Committee's advice for public information and debate. The first advice, released in May, commented with fulsome praise on President Carter's message to Congress on energy. It said that, despite obvious differences arising from the levels of consumption, "the basic conservation principles expressed in President Carter's statement are, in the Committee's view, directly applicable to the Australian situation". It advocates, for Australia, smaller cars, more effective use of public transportation, reduction of wastage, use of surface rather than air transport, rail rather than road transport where appropriate, and lowering speed limits on open roads. As with the Fox report, the government has given no indication of action to follow this advice.

Peter Pockley



Mary Kathleen uranium mine, circa 1961

Astronomy's own cloud

Meteorology and astronomy, two areas which seem to boil over periodically and publicly, have been placed under inquiry by the Australian Department of Science. Both committees of inquiry have been under way since late last year and were charged with reporting to the Minister for Science, Senator James Webster.

Neither report has been completed nor been made public. Some anxiety on this point is evident. The Secretary of the Department of Science, Sir Hugh Ennor, has only a few months left before retirement and, being the forceful character that he is, he doubtless wants to wrap up his work with the weather and star men safely settled for a while.

Relations between the Australian Bureau of Meteorology, headquartered in Melbourne, and the Canberra-based Department of Science have been tense ever since the bureau was transferred to the Department's responsibility under the Labor government. The committee was set up following regular and damaging leaks in the press of alleged confrontations between the bureau and the department. Its terms of reference included "making recommendations on the Head Office and Regional Organisational structure", but other terms cover all the bureau's functions.

The bureau has operated under its present act, the Meteorology Act, since 1955. The accompanying degree of independence was important to its Director, Dr Bill Gibbs, and to many users in this vast land mass where the rural industry and aviation services are peculiarly dependent on sound and comprehensive weather advice. The inquiry was seen by many as being the only method of resolving the bureaucratic conflicts. Significantly, the three-man committee did not include any professional meteorologist.

Things are a little different in astronomy. Ever since the early 1960s when Australia leapt into the big league of astronomical nations with the building of the giant 64-metre radio dish at Parkes, New South Wales, a clamour for large facilities has been maintained, and to a reasonable extent satisfied. From the astronomer's point of view, their requests made good sense. Despite its relatively low terrain, Australia provided the only geologically and politically stable platform in the southern hemisphere for observing stars obscured to northern telescopes.

To a certain extent the argument of uniqueness is no longer as strong

following the construction of some large telescopes in the South American Andes and Hawaii—at which stage other arguments take over, about maintaining a world lead, keeping the best scientists at home, and the prestigious PR that a nation gets from big telescopes. To back these arguments, the Australians now have a magnificently timed and very visible example of how astronomical research can lead to necessary (and profitable) application on Earth. At the end of March the Australian-developed 'Interscan' system for aircraft landing control in three dimensions was officially adopted as the next international standard. Interscan beat all comers from the USA, the USSR, Britain and Europe and is now the basis of a burgeoning industry. The microwave system was developed by the radioastronomers of the CSIRO Division of Radiophysics.

The Parkes facility was expanded in the mid-1960s to become an interferometer, and the main dish has recently been refigured at its centre to accept millimetre wavelengths. Among the other facilities around the country, the University of Sydney built its own radio interferometer—the so-called 'Mills Cross', named after its designer, Professor Bernard Mills—and an optical interferometer, Professor Robert Hanbury Brown's Stellar interferometer at Narrabri in northern New South Wales (now at the end of its useful life). CSIRO's Division of Radiophysics opened a magnificent solar observatory at Culgora, also in northern NSW, with Dr Paul Wild's Radioheliograph, together with optical solar telescopes of another division. Meanwhile, the Australian National University was expanding its optical observatory at Siding Spring in northern NSW, and in 1974 the joint Anglo-Australian Telescope Board opened its 3.9-metre telescope on the same mountain, then the largest optical telescope in the southern hemisphere. Beside it, the British also now operate a 1.9-metre Schmidt sky camera.

Enough for a nation of nearly 14 million people and few astronomers? Not so, said the astronomers, and asked for more—here remains our one chance of maintaining across-the-board, world-class excellence in a branch of science. Because of the sums involved for new facilities of significance, leading astronomers the world over have had to become politicians, and the Australians are no dullards at this game. Every minister responsible for science in Australia

has eventually cried for mercy—continuance of the ad hoc lump-sum handouts of earlier days could no longer be justified, they said, without the unanimous recommendation of an expert committee.

And so began an unceasing stream of references from ministers to committees which either never reached unanimity or had to be replaced because of political change. The latest act has been the establishment of yet another committee to end all committees, this one by Senator James Webster.

The committee, it was announced, was to be "an interdepartmental committee (IDC) chaired by the Department of Science. The Committee comprises representatives of the Public Service Board and the Departments of Education, Science and the Treasury". The IDC (of non-astronomers, it will be noted) was charged with inquiring into the range of astronomical research and facilities.

The public servants of the IDC would clearly be responsible for making the recommendations to the minister. However, an "expert sub-committee" was named to assist the IDC. And there was the rub, as far as the scientific community was concerned. For, while the names of the sub-committee were publicised (it is chaired by Professor Kevin Westfold of Monash University), the IDC remained anonymous in the official announcement.

The gentle inquirer, however, would soon be told that the IDC was chaired by the redoubtable Sir Hugh Ennor, and included the well-regarded Secretary of the Department of Education, Mr Ken Jones, and some named but lesser fry. Yet the very possibility of some private shenanigans continued to worry some participants. The influential Academy of Science is understood to have initially refused to make a submission over the Academy's name.

As far as the astronomical politicians were concerned, an IDC was quite clearly a most inappropriate vehicle for assessing their aspirations. For the government, however, it has kept the lid on some vocal people for a while. Yet the government has let itself in for at least one more round by announcing that the IDC's report (intended for the Minister for Science) would be referred to ASTEC "for comment". And ASTEC reports to the Prime Minister, who is charged in most cases, and certainly including this one, with releasing their reports.

Peter Pockley

The federal Inquiry into Education and Training, which is trying to relate post-secondary education and the job market, will also impinge on science, though not directly. Professor Bruce Williams, an economist who is Vice-Chancellor of the University of Sydney, is chairing the Inquiry which is being taken very seriously indeed by the three tiers of universities, colleges of advanced education, and technical and further education.

If the current political cry for 'relevance in education' (that is less unemployment among secondary and tertiary education leavers) has any pull left by the time the Inquiry reports next year, then a reduction in the research and scholarship activity at universities relative to vocationally oriented training is possible. However, the appointment of a resilient university man as chairman is reasonable protection for the university position.

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Siding Spring Observatory (see opposite)

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Parkes radiotelescope (see opposite)

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ASTEC about its continuation*. Mr Fraser's statement is worth quoting at some length:

The functions of ASTEC are to advise the Government on science and technology, including

- the advancement of scientific knowledge and the development and application of science and technology in relation to the national well being;
- the adequacy, effectiveness and overall balance in the national effort in science and technology in Government, industry, education and other sectors of the community;
- the assessment of gaps and overlaps in science and technology in Australia;
- the identification and support of new ideas of science and technology likely to be of national importance;
- the practical development and application of research discoveries and the fostering of technological innovation in industry; and
- the means of improving efficiency in the use of resources related to science and technology.

The Council will have a strategic role in assisting the Government to encourage Australian science and technology to meet the nation's needs and objectives. It will have no executive responsibilities but will be able to advise on operational arrangements.

ASTEC's knowledge and analysis of science and technology will be valuable to many arms of Government. And the Government expects the Council to inform itself and be informed of relevant Government policies and to take into consideration economic and budgetary implications in discharging its functions.

ASTEC will draw on existing Departments and Agencies for the expertise, knowledge and assistance necessary to enable these functions to be discharged effectively. But this will in no way compromise the independence of ASTEC.

ASTEC has been charged first with preparing a long-overdue report on the present state of science and technology in Australia. Information about Australia's effort has been so fragmented, particularly in the government arena which dominates national R&D expenditure, that the very thought of deriving coherent policies from such information boggles the mind. This task may well be measured in years and is almost certain to out-run the life of the present Parliament, which has only 18 more months at the most.

ASTEC is thus unlikely to influence the science funding sections of the August 1977 budget, framing for which is already well advanced (to cries of "horror budget cuts"), and may hardly have sufficient time to influence the

1978 budget and the present government's final burst of legislation before facing the people again.

As anticipated in earlier reports, the permanent ASTEC reports to the Prime Minister. Its small secretariat is established within the Prime Minister's own Department. That these moves will be popular among scientists and technologists is quite certain. An analysis of the views expressed in submissions to the Interim ASTEC showed that 46 of them favoured ASTEC reporting to the Prime Minister, 6 to a Ministerial Committee, 3 to Cabinet as a whole, 4 'others', and a bare 2 to the Minister for Science. Hardly a resounding vote of confidence in the political clout of the scientists' 'own' Ministry.

The government intends that ASTEC's reports will be made public "unless there are overwhelming reasons in the national interest for not doing so" (in other words, when the security boys cry stop). Meanwhile the Australian Academy of Science, whose influence over government went into a small, temporary decline under Labor, has emerged triumphant with the appointment of its current president, Professor Geoff Badger, as part-time Chairman of ASTEC, and its immediate past president, Professor Sir Rutherford Robertson, as Deputy Chairman. Professor Badger, an organic chemist, is now a research professor at the University of Adelaide, having recently completed a ten-year stint as its Vice-Chancellor. He is firm, persuasive and skilled at the kind of politicking ASTEC needs for success.

While it was officially said that members of ASTEC are not selected as representatives of particular interest groups, it is not hard to see the influence on the present government of the major areas of Australian industry. The rural, steel, mining, chemical, electronics and paper industries all have senior executives on ASTEC, including three managing directors. Just how much time such busy men (there are no women among ASTEC's fifteen members) can devote to the detailed collection and assessment of information across the whole spectrum of science remains to be seen.

In theory, there appear to be few restrictions on ASTEC's coverage of the science and technology scene. The Interim Council's report set itself against covering the social and behavioural sciences, but argued for the inclusion of both the defence and health sciences. In Labor's terms of reference for an earlier Interim ASTEC, social science was in, but defence and health were out. Whether these items will be specified in the legislation is not known as yet. □

ALS, London

ALS, London

*Future Arrangements for an Australian Science and Technology Council, Report to the Prime Minister by the Interim ASTEC, November 1976 (Australian Government Publishing Service; 1977).

correspondence

Scientists think too

SIR,—Thank you for referring to my recent Presidential Address to the American Physical Society (28 April, page 759). However the irony implicit in your references to my address does not escape me. I disagree with you especially when you closely couple some of my words to “the recent frenzy to project science as still valuable”.

Come off it. On rereading my address and listening to a recording I find nothing frenetic about it. In fact I delivered the address in an old sweater. I figured that a President of the American Physical Society, nicknamed Willy, was not to be outdone by a President of the United States, named Jimmy. The tape tells me I got some laughs but mainly I was in dead earnest. Sure, scientists are thinkers but our public image is just that—and long hair too! If society is to reach a solution of its present horrendous problems it must permit the scientist to be more than thinker, consultant and adviser. It must let him be a doer in the sense of executive, manager, legislator all the way to trouble-shooter and fix-it man. Society seems to have forgotten what happened in the winning of World War II and is now wasting a potent resource. You delude the public and discourage students by harping on the image of the scientist as the thinker. It obscures the role the scientist can perform as man of action.

WILLIAM A. FOWLER

Past President,
American Physical Society,
Caltech, California

SIR,—While agreeing with the sentiment of your advocacy for the role that could be played by scientists in the affairs of the nation (28 April, page 759), your suggestion that the mass media should take it upon itself to act as a public relations vehicle for science is both a serious indictment of scientists, and a misconception of the role of the media.

Science is undoubtedly the most highly funded cultural activity of today, yet precious little if any of this funding is devoted to developing the communication talents of its practitioners. British scientists, in my experience as a programme producer, are no more nor less talented as broadcasters than any other group of pro-

fessional people. They are certainly no more nor less perceptive about the affairs of the nation than say lawyers, historians, philosophers. Your proposal that the media create an image for scientists seems to be asking that the media take part in a cover-up campaign. You're asking us to hide a failure in the training of scientists.

You write that “very few scientists are allowed to express their own views in any extended way”. I could quibble with your words “very few”, but I would like to draw your attention to some science broadcasts you appear not to hear. For example, the fortnightly *Scientifically Speaking* on Radio 3 is the kind of programme you say is missing. In a year's broadcasts about 30 notable scientists (not to my mind “very few”) take part usually in a 45 minute conversation. Through the questions of the interviewer, even the totally uninitiated yet intelligent listener is taken to the epistemological frontiers of science. Sydney Brenner, Leon van Hove, Nick Humphrey, D. T. Whiteside and B. F. Skinner—all participants in recent editions of *Scientifically Speaking*—falsify your general proposition that radio does badly in portraying the scientist as thinker. In broadcasting, what achieves impact is high programme standards, not saturation of the wavebands with merely adequate broadcasts mounted to present an image.

There also exists on Radios 3 and 4 another admirable vehicle for scientists as homo ‘sapiens’—the scripted talk. I am not inundated with unsolicited ideas. You might well argue that we are too restrictive in allowing speakers the freedom to choose their topics. I would counter by drawing your attention to the long running Radio 3 series *Personal View*, transmitted on alternate Saturday evenings. In the course of four, 20-minute *Personal View* talks, the speaker is encouraged to reflect on topics of his own choice. Over the past three years, the three scientists who have given *Personal View* series have set a high standard for the others to come. Dauntingly, it seems, because many of those approached are put off by a brief that asks them to argue on topics that are in the public eye.

Surely scientists do have views, but why are they so often inhibited when offered a platform to expose their thoughts on issues outside their field of

professional enquiry? Let's not have image-building. Broadcasters are not public relations men; let's have the real article.

DAVID PATERSON

BBC, London, UK

T. H. Huxley, a self portrait

SIR,—I found this self-portrait of T. H. Huxley among the effects of Mr W. D. Bradfield, assistant to Sir William de W. Abney, a former physics professor at Imperial College, best known for his researches in colour vision.

In his autobiography T. H. Huxley states that he inherited his faculty for drawing from his father but never received any formal training. Sir Julian Huxley considered his grandfather's sketches almost as finely executed as those of Leonardo da Vinci.

Bradfield photographed the drawing and the back of the photograph reads “photograph of pencil drawing of Professor Huxley. Drawn by Professor Huxley in Captain Abney's memo book”. Abney joined the Science and Art Department, South Kensington in 1877 and Huxley resigned his appointment at the Royal School of Mines in 1885. The sketch was therefore probably drawn sometime during this period when Huxley was in his mid-fifties.

M. DAVIES

University of Surrey,
Guildford, UK



news and views

The protein explosion

from Denis Bray

THOSE cell biologists who feel that the flux of information in their subject is already too high will view the latest development in gel technology with mixed feelings. For where the typical SDS gel of two years ago had some 20 well resolved bands, the two-dimensional gels that are presently *de rigueur* for the analysis of protein mixtures have several hundred. The amount of information obtained from one sample is thus appropriately, squared. The new method, devised by O'Farrell (*J. biol. Chem.* **250**, 4007; 1975), consists in electrophoresis in the presence of urea and ampholines in one dimension, and in SDS in the second. The proteins move into positions in a two-dimensional array determined by the independent parameters of isoelectric point and polypeptide size.

The system is potentially able to resolve 7,000 proteins, and although in his paper O'Farrell modestly admitted to having achieved only 1,100 from a bacterial lysate, the limitation is more likely to be in the *E. coli* genome than in his technique. From the rapidity with which it has been exploited the method obviously satisfies a long-felt need, and for many purposes it replaces a number of more complicated methods in which protein bands from gels are identified by proteolytic or chemical cleavage. It has already been widely applied and has shown even familiar biological materials to be more complicated than anyone had imagined—the flagellar axoneme for example, has over 100 components (Piperno *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 1600; 1977). The worry that the electrofocusing step might generate artefactual spots may have existed at the early stages but has gradually disappeared with use. It is explicitly disproved by such striking demonstrations as that of Milman *et al. (Proc. natn. Acad. Sci. U.S.A.* **73**, 4589; 1976), that a single genetic defect in HeLa cells affects only one spot on a 2-D gel. One is forced to conclude that each spot corresponds to one protein, and in consequence that

there are a very large number of proteins about which we know nothing at all.

Another, ostensibly unrelated, way to examine proteins is with fluorescent antibodies. This established procedure, developed by Coons more than 35 years ago, received its cell-motility debut quite recently with the isolation of anti-actin and its use to show microfilament bundles within fibroblasts (Lazarides & Weber *Proc. natn. Acad. Sci. U.S.A.* **71**, 2268; 1974). Since then, chorus-lines of glittering actin, myosin, filament protein, or LETS protein—to name a few—have brightened the biological journals in such routines as mitosis, transformation or colchicine treatment. Each pattern is characteristic of the protein and situation, even—according to the provocative suggestion of Albrecht-Bühler (*J. Cell Biol.* **72**, 595; 1977)—to the point of showing familial resemblances between daughter fibroblasts. The technique is not restricted to major protein components; indeed, because its sensitivity is uncontrollable even trace contaminants can give a sizeable response. Just how sensitive the method is will depend on the precise conditions, but some idea may be gained from the fact that a few hundred thousand antigen molecules on a lymphocyte surface give an adequate response. From this it should be possible to see 1,000 molecules of an antigen on a small area.

The two approaches of gel electrophoresis and immunological localisation have often been combined, in two recent reports of elegant work on muscle for example. Antibodies to C-protein (named for its position on an SDS gel) were prepared by Craig and Offer (*Proc. R. Soc. B* **192**, 439; 1976), and found to bind nine transverse stripes on either side of the A-band of striated myofibrils. That is, nine stripes were labelled in fibrils close to the edge of the muscle fibre, but only seven or eight in fibrils closer to the centre. The difference was revealed as being due to two other antigens in the original C-protein mixture, and by absorption, one or two stripes of the original nine could be stained. An even closer relationship between the techniques exists in recent

work on the 100 Å filaments of smooth muscle. These were recently prepared in good yield by Cooke (*J. Cell Biol.* **68**, 539; 1976), and by Small and Sobieszek (*J. Cell Sci.* **23**, 243; 1977), and are made of a protein with an apparent molecular weight on SDS gels of about 50,000. If this band is excised and the protein it contains injected into a rabbit, antibodies are obtained that stain not only smooth muscle, but the Z-disc and inter-fibrillar regions of striated muscle, and the areas of cell-to-cell contact in heart (Lazarides & Hubbard *Proc. natn. Acad. Sci. U.S.A.* **73**, 4344; 1976). When it is examined on 2-D gels this protein reveals two components which seem to be the same regardless of the muscle type. (Izant & Lazarides *Proc. natn. Acad. Sci. U.S.A.* **74**, 1450; 1977).

The synergism between gels and antibodies illustrated by such work is impressive but short of the ultimate. For surely it is entirely feasible now to extract proteins from large 2-D gels, raise antibodies to them, and use these to locate and purify the native protein? The possibilities then become awesome. If a technique in which a thousand proteins can be resolved is combined with one that can detect a thousand molecules, it could be like bringing two sub-critical masses of fissionable material together. What then would limit the isolation of new proteins, and how many biochemists would have to be applied, and for how many years, before the end was reached? Perhaps it will depend, ultimately, on how many proteins there are in all. The thousand spots seen by O'Farrell in extracts of *E. coli* must have represented the lion's share of that organism's proteins and if mammalian cells have of the order of 10,000 proteins—in keeping with the genetical estimates of 5,000 for *Drosophila* and 2,000 for *Caenorhabditis*—then there will be some hope of a complete account. But what if the geneticists are wrong and most of the potential for 100,000 proteins in the non-repetitious DNA is expressed? And what if someone invents a 3-D gel? Then, surely, it will be time to call a halt and to show the responsible attitude recently developed towards plas-

mid research. Perhaps the discovery of new proteins could be confined to rigorously controlled (P4) conditions, or a two-year moratorium on gels could be held. At least that would let us catch up with the literature. □

Metabolic studies using ^{31}P NMR

from G. E. Chapman

THE state of proton NMR spectroscopy of biological macromolecules is somewhat akin to that of X-ray crystallography before methods of solving the phase problem had been devised. There is an embarrassment of detail, with as yet no reliable methods of interpreting it in terms of molecular conformation, though it is relatively simple to work backwards from a known structure to the observed spectrum. It is not surprising therefore that much effort is now being directed towards the NMR study of those heavier nuclei where there is less detail in the spectra, and interpretation is easier.

The increased use of ^{31}P NMR in biological research in the past year or two owes much to magnet development. In the continuing search for greater sensitivity, the NMR spectrometer designer has usually opted for higher magnetic field strengths, stretching superconducting magnet technology to the limit. However, there are indications that increasing magnetic field strengths are not achieving increases in sensitivity for the heavier nuclei when they are incorporated in macromolecules. Hence one alternative development has been that of magnets capable of maintaining very homogeneous fields over large sample volumes. This is particularly advantageous where the sensitivity is limited by the concentration of the sample rather than by the amount of material available. The following recent work has been made possible by these developments.

The monitoring of phosphate metabolite levels in living cells and tissues by NMR, being non-destructive, is aesthetically more pleasing than the conventional freeze-clamp techniques that have been used in the past by biochemists. However, it could be argued that both methods give the same results, and that a great many freeze-clamp experiments can be done for the price of buying and maintaining a modern advanced NMR spectrometer.

This question probably cannot be resolved at present. A hybrid of the two methods has recently been illustrated, where the phosphate metabolites from the extract of a quick-frozen heart were analysed by ^{31}P NMR (Garlick *et al. Biochem. biophys. Res. Commun.* **74**, 1256; 1977).

One type of measurement by ^{31}P NMR in living cells however is giving results which have not been obtained reliably and accurately by other methods. This is the measurement of intracellular pH. It is achieved by observation of the inorganic phosphate chemical shift, which changes with pH in the region of neutrality due to its second acidic dissociation, and is capable of considerable accuracy (better than ± 0.02 pH units near pH 7). The intracellular pH of living *Escherichia coli* suspensions has been measured in a variety of metabolic conditions (Navon *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 888; 1977). It was shown that the intracellular pH was consistently higher than that of the external medium during respiration, but if respiration ceased, then the pH values rapidly equalised. This supports the hypothesis that an outward directed proton pump mechanism operates during respiration. Experiments on chromaffin granules (the catecholamine-storing vesicles of the adrenal medulla) by ^{31}P NMR also used chemical shifts to detect pH changes within the vesicles (Casey *et al. Biochemistry* **16**, 972; 1977) though in this case the resonance monitored was the γ phosphate of ATP rather than inorganic phosphate.

Although *in vivo* experiments are inherently more spectacular than *in vitro* ones, from the view point of the biologist, the latter type of study can be better controlled. Investigations of the important metabolic regulatory enzyme system glycogen phosphorylase, is a classic example of the importance of *in vitro* studies of metabolic processes. There are at least five phosphate compounds associated with the enzyme as substrate, cofactors and allosteric effectors. It has been investigated by ^{31}P NMR in the past, but the high molecular weight has meant the use of very high protein concentrations to achieve adequate molar concentrations of the bound phosphates, which in turn has given rise to considerable viscous broadening of the resonances. This has recently been overcome by the use of large sample volumes, and in the latest paper (Feldmann & Hull *Proc. natn. Acad. Sci. U.S.A.* **74**, 856; 1977) the role of pyridoxal phosphate in the enzyme complex has been investigated. In such an NMR experiment, one would expect some of the resonances to overlap, and the authors overcome

this by the ingenious device of using thiophosphate analogues, where these were known to be active in the enzyme system. Thiophosphates have chemical shifts about 40 p.p.m. downfield from those of the corresponding phosphate compounds. Hence the resonances of all the phosphates could be resolved. In this way, much was learnt about the interaction of the molecular species involved.

The above exemplifies, I believe, how good communication and teamwork between the biochemists and physical technique specialists can advance the application of a technique to biological problems. Here the interdisciplinary approach is paying handsome dividends. □

The nucleotide-binding fold revisited

from C. C. F. Blake

SOME time ago in these columns (*Nature* **250**, 284; 1974) I discussed the significance of a particular protein fold that had been noticed by Rossmann to constitute part or all of the structures of four NAD-dependent dehydrogenases and a number of other enzymes, some but not all of which also bind nucleotides. In lactate-, alcohol-, and glyceraldehyde-3-phosphate dehydrogenase, the fold corresponds to a separately organised NAD-binding domain, that in each molecule is linked to a second domain whose tertiary structure is specific to the particular enzyme. On the basis of this structural pattern Rossmann and his colleagues (*Nature* **250**, 194; 1974) proposed that the NAD-binding domains had evolved by divergence from a common ancestor, and had been incorporated into the dehydrogenases by gene fusion with another protein fragment, different in each enzyme, that became responsible for that enzyme's particular catalytic and substrate binding properties. Potentially this proposal provides a profound insight into the evolution of those groups of enzymes that catalyse the same type of reaction, often using common cofactors, but working on quite different substrates. The key to the proposal is the assumption that an extensive structural similarity, unsupported by sequence homology, represents the relic of a very distant gene duplication. However, so little is known about the factors controlling protein

structures that it is very difficult to draw the essential distinction between homology and analogy. Since in the past few months three papers have appeared that in various ways bear on the problem of the significance of the nucleotide-binding fold, it seems worthwhile to return to the topic.

Describing the tertiary structure of phosphorylase α , Fletterick *et al.* (*J. biol. Chem.* **251**, 6142; 1976) note that a part of its 800-residue polypeptide chain is folded into the nucleotide-binding pattern, and demonstrate its close similarity to the NAD-binding domain of lactate dehydrogenase. In agreement with the prediction of Rossmann's proposal, Madsen and his colleagues have observed that the fold is the location of a binding site for adenine or adenosine that is close to the site for the adenine ring of NAD in the equivalent region of lactate dehydrogenase. Unfortunately the significance of this binding site is not known, and the site for the known allosteric effector AMP, is elsewhere in a region whose structure is quite different from the nucleotide fold. Nevertheless, the discovery of the fold in yet another enzyme, associated with nucleotide binding, must strengthen the hypothesis considerably.

One of the features of the fold that was instrumental in its discovery was that the usually helical loops that sequentially link together the six-stranded parallel β sheet, were all arranged in a right-handed sense, thus giving rise to a characteristic distribution of connections above and below the sheet. At the time both right- and left-handed turns were thought to be allowed, suggesting that the repeated occurrence of a particular pattern in the disposition of the connections was highly significant. Sternberg and Thornton (*J. molec. Biol.* **105**, 367; 1976) have now shown that this view is incorrect. By analysing all known proteins in terms of the disposition of the loops linking adjacent β strands, they have found that in 57 out of 58 cases the connection has a right-handed sense. Furthermore they have produced a soundly based argument that the right-handed connection is a natural consequence of the steric factors imposed by the twist of the β sheet, which Chothia (*J. molec. Biol.* **75**, 1295; 1973) has shown, can itself be understood in terms of the interactions within the individual strands of the sheet. This means that the five right-handed connections in the nucleotide-binding fold are not independent parameters, with which the fold can be defined, but are the consequence of good molecular packing. The removal of these parameters has dramatically reduced the figure of topological relatedness calculated by Schulz and

Schirmer (*Nature* **250**, 142; 1974) for a number of proteins in which the fold seemed to occur, to leave only the topologies of the cofactor-binding units of the dehydrogenases with a sufficient degree of relatedness to need a special explanation. Rossmann's hypothesis still stands then, stronger I suspect for having a number of dubiously-related enzymes stripped away from it by Sternberg and Thornton, leaving a solid core of structure whose relations have a firm functional foundation.

The most striking new addition to the catalogue of enzymes showing tertiary structural homology is pyruvate kinase, whose structure has been recently reported by Stammers and Muirhead (*J. molec. Biol.* **112**, 309; 1977). The structure of the 60,000 molecular weight subunit is divided into three domains, the largest of which is closely similar to the complete triose phosphate isomerase (TIM) subunit. As described by Banner *et al.* (*Nature* **255**, 609; 1975) the TIM subunit has a hitherto unique structure composed of eight parallel β strands forming a closed barrel in the core of the molecule, surrounded by helical connections, giving it an unusually simple, symmetrical organisation. This structure is sufficiently characteristic for there to be little doubt that it constitutes the central domain of pyruvate kinase. The active site of pyruvate kinase is located in an equivalent position to the active site of TIM, in relation to the common structure. These structural and functional similarities between pyruvate kinase and TIM are of the same order as those in the various versions of the nucleotide-binding fold, and should therefore in principle be treated in the same way. Is it then plausible that pyruvate kinase and TIM should have a common ancestor? The only thing that one can say is that despite the quite different kind of reaction catalysed by the two enzymes, their substrates are both 2-keto-trioses. Whatever arguments are applied to the significance of the nucleotide-binding fold; it seems that they must now also bear on the 'keto-triose fold' and *vice versa*; they stand, or fall, together.

Finally the structure of pyruvate kinase focuses attention on the very curious fact that while the four dehydrogenases whose tertiary structures are known show a significant degree of similarity to one another, the four kinases do not. Adenylate kinase is somewhat similar to a part of phosphoglycerate kinase (PGK), but does not seem to be significantly related; PGK itself almost certainly contains at least one, if not two, copies of the nucleotide-binding fold; pyruvate kinase is structurally related to TIM as we have seen; and hexokinase seems

to have a unique structure. The search for indications of enzyme families through structural similarities will continue, but it seems that any pattern that emerges is unlikely to be simple and clearcut. $\square$

Systematic behaviour of E1 decays in sodium

from P. E. Hodgson

THE electric dipole (E1) transitions in light nuclei have so far received little theoretical attention because selection rules often forbid transitions between the major components of the wavefunctions of the initial and final states, so that they depend on small components of the wavefunctions which are difficult to calculate. In spite of this, the experimental E1 transitions in ^{23}Na show a remarkably simple pattern that is quite different from that given by the simple rotational model. Recently Pilt has developed an alternative model that agrees very well with the experimental data for ^{23}Na and also for ^{21}Na . (*Z. Naturforsch* **32a**, 73; 1977).

The E1 decays in ^{23}Na take place between the lowest $1/2^-$ band and the ground state $3/2^+$ band. It is found that when the overall E^3 dependence of the transition rates is removed a spin J state of the $1/2^-$ band decays preferentially to a spin $J+1$ state of the $3/2^+$ band. A completely different behaviour is predicted by the simple rotational model, which gives reduced transition strengths proportional to the squares of the appropriate Clebsch-Gordan coefficients.

It is most likely that such regular deviations from the simple rotational model are due to an interference effect, and Pilt suggests that this can be provided by mixing higher Nilsson orbitals into the ground state band. The most likely orbitals are those with spin and parity $1/2^+$ and those with the configurations [220] and [211] are the most probable. The E1 transition rate is then given by the absolute modulus squared of the sum of the appropriate Clebsch-Gordan coefficients, multiplied by weight factors, that is,

$$B(E1) = |(J1/2 \ 11/J'3/2) + m_1(J1/2 \ 10/J'1/2) + (-)^{J'-1} m_2(J1/2 \ 1-1/J'-1/2)|^2,$$

where J and J' are the spins of the initial and final states, and m_1 and m_2 are the weight parameters which are related to

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the degree of mixing between the $1/2^+$ and $3/2^+$ bands.

So far it has not been possible to calculate the mixing parameters m_1 and m_2 , but the model may be tested by using the experimental values of $B(E1)$ for two transitions to determine m_1 and m_2 , and then to use these values to calculate the $B(E1)$ values for the other transitions. Pilt did this for the transitions in ^{23}Na , and found that the reduced transition rates for the whole band are very well given by this model. The same analysis was applied to the transitions in ^{21}Na , and this also gave good overall agreement with the data, although it did not have such a simple pattern as that for ^{23}Na .

An attempt to calculate the mixing parameters from the Nilsson model was not successful; it was found that there is no value of the nuclear deformation that gives the observed distribution of transition strengths. It thus seems that a more detailed microscopic theory is needed to obtain a proper understanding of these quantities.

Even in its present form, with the parameters treated as adjustable and fitted to the data for each nucleus, the model can be very useful in correlating experimental data, and it could well be a useful method of determining the spins of high-spin states. It would clearly be useful to test its applicability to other nuclei, and this might show some systematic behaviour of the mixing parameters, which in turn might provide a clue to their microscopic interpretation. $\square$

A tribe of mimics

From a Correspondent

BLENNIES are generally known as small, rather retiring fishes living in shallow water close to rocks or coral. Very many species are known in temperate and tropical seas but the latter contains the greatest number of species. A few are found in freshwater. Partly because there are so many nominal species, ichthyologists were exercised in attempting to assess their phylogeny, and for long the standard work on the group as a whole was J. R. Norman's provisional synopsis (*Ann. Mag. nat. Hist.*, ser. 11, 10, 793-812; 1943). More recently, V. G. Springer's study of the osteology and classification of the family (*Bull. U.S. natn. Mus.* 284, 1-85; 1968) has provided a major clarification and a useful overall classification within which Springer, and others, can tackle the smaller groups in the family.

The most recent is a revision of the sabre-toothed blennies of the tribe Nemophini by W. F. Smith-Vaniz (*Acad. natn. Sci. Philadelphia, Monogr.*

Origin of North China loess

from I. J. Smalley

CHINESE civilisation developed on the great North China loess deposits. Ho (*Cradle of the East*, Chicago University Press, 1975) has argued convincingly that this development took place in isolation, uninfluenced by the parallel progress in the Middle East. A major factor which did influence the growth of the early Chinese civilisation was the presence of the loess which was an ideal soil for the early farmers. The origin of this North China loess is still in dispute. Most of the other loess deposits in the world have glacial connections and their formation can be seen as part of a sequence of Pleistocene events, but the Chinese loess does not have an obvious glacial link.

The results of the first electron microscope observations of Chinese loess have now been published (Lu *et al. Geochimica* 47, 1976). These allow the Chinese workers to reject totally the Berg theory of *in situ* formation, but they still find themselves unable to accept totally a direct glacial connection. The grains observed are broken and have sharp edges, and silica precipitation and chemical etching are commonly observed on the quartz silt particles, which might have been subjected to relatively long weathering processes

before being transported to the loess accumulation areas. In the relevant areas the authors suggest that glaciation only occurred on mountain tops, and that this may have produced some loess, but they favour a distant western source for the majority of the material. No specific particle-producing mechanism is described.

It may be that in this case a longer than usual transportation stage was involved and that material was formed in distant glaciated regions and that the continuing aridity and the presence of mixed particle deposits in the post-Pleistocene times prevented the formation of stable loess deposits. In Europe and North America loess deposits form very close to the ice limits. The Lu *et al.* paper is an encouraging sign of the renewal of interest in loess sedimentology in China. It is a pity that those otherwise admirable data handlers, *Science Citation Index* and *Mineralogical Abstracts*, have not realised that in Chinese the family name (as used in references) comes first; so this important paper has been listed under Yanchou *et al.* which could cause some confusion.

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19, 1-196; 1976); an excellent contribution to the systematics of these Indo-Pacific blennies. As a taxonomic work it is of fundamental importance for not only are 41 species in 5 genera fully described and figured, but 13 of these species are new taxa, recognised here for the first time. However, this paper is important not just because it is a highly competent revision, but because of the information about the life styles and behavioural relationships of this fascinating group of blennies.

The sabre-toothed blennies are so called because they all have a massive recurved canine tooth posteriorly in the lower jaw. Most species also have smaller caniniform teeth in the upper jaw. In the front of both jaws the teeth are small, closely-packed and incisiform. Such dentition suggests that their feeding behaviour might be specialised, an expectation which is fully realised.

Members of the genus *Meiacanthus* are unique among fishes in having well-developed toxic buccal glands. Their sabre teeth are grooved, the groove leading to the gland in the dentary bone and acting as a channel for the pale, toxic secretion. All the species of *Meiacanthus* bite when attacked, and

experiments have shown that they are generally unacceptable as prey although naive fish predators may take them into their mouth, only to spit them out quickly. In ecology and behaviour they differ strikingly from most blennies. They inhabit open water and in daytime forage over a wide area in search of small crustaceans in the water column, and tend to hover in one spot then jerk away, to hover again, feeding as they pause.

Their virtual immunity from predators no doubt has a bearing on their behaviour and it is not surprising that several species are brilliantly coloured in life. Various *Meiacanthus* species have close mimics, including unrelated fishes, as well as other sabre-toothed blennies.

The latter include several species of *Plagiotremus* which hover and jump in much the same way as the *Meiacanthus* model. The former feed on larger fishes, especially non-predatory ones, hovering nearby and then launching a lightning attack on the passing fish, usually from the rear. If the prey turns head-on suddenly the attack will be aborted. Their incisor teeth are curved, sharp in the horizontal plane, but flattened in the lateral, are well adapted

to slice skin and scales out of their prey, while the massive fangs provide a purchase as well as breaking the surface tissues. In all the specimens which contained food, Smith-Vaniz found mucus and dermal tissue.

A member of a third genus of sabre-toothed blenny, *Aspidontus*, is also an accomplished mimic, but this time of the cleaner wrasse, *Labroides dimidiatus*. *Labroides* is cryptically coloured and tends to hide when approached. *Aspidontus taeniatatus* employs its resemblance to the generally unmolested cleaner wrasse to approach naive prey and attack them, biting off fin rays and associated membranes.

Petroscirtes, the fourth genus of sabre-toothed blenny also includes at least two mimics of the venom-fanged *Meiacanthus* species, but most of the 10 species recognised by Smith-Vaniz are retiring and cryptically coloured. They feed on copepods and diatoms and are thus rather unremarkable as

these blennies go. However, for small fish (they rarely grow longer than 60 mm in body length) they are well able to defend themselves, even by snapping at probing fingers.

To complete his account of the nemophine blennies, Smith-Vaniz describes the two species of *Xiphasia*. True to the nonconformist nature of the tribe, they differ from most blennies. They may attain a length greater than 500 mm, so they are exceptionally large members of the family. They are also semi-pelagic and probably nocturnal, and it seems that they burrow tail first into sand or mud, but their biology is not yet fully studied.

Smith-Vaniz's revision of this tribe of blennies, although primarily a taxonomic treatment, shows them to be animals of striking behavioural and ecological divergence. This group, at least, are far from the dull-coloured, retiring, and modest animals that one generally thinks of as blennies. □

Antibiotics and bacterial sporulation

from Brian N. Dancer

It has been known for many years that bacteria of the genus *Bacillus* produce antibiotic substances when the cells are beginning endospore formation at the end of growth. These compounds consist of oligopeptides often containing non-protein amino acids and they are not synthesised on ribosomes. They are a heterogeneous group of compounds some of which (such as gramicidin) are surface-active agents whereas others (bacitracin for example) inhibit cell wall synthesis. For some time there has been controversy over their function. Secretion at the end of growth has been seen as a mechanism for inhibiting competitors, and allowing greater growth by the producer organism. Alternatively, antibiotics may be secondary metabolites synthesised to remove accumulated toxic compounds. A third view is that their production at the beginning of sporulation indicates a role in the regulation of the developmental process. If the last were true one would expect that cells deficient in antibiotic synthesis would sporulate abnormally. This hypothesis has recently been tested by Mukherjee and Paulus (*Proc. natn. Acad. Sci. U.S.A.* **74**, 780; 1977) in *Bacillus brevis*, a strain which produces two classes of antibiotic, gramicidins and tyrocidines. The authors were able to select mutants using an ingenious technique developed

by Raetz (*Proc. natn. Acad. Sci. U.S.A.* **72**, 2274; 1975). These mutants are unable to make gramicidin although tyrocidine is still produced. They are able to sporulate but the spores are somewhat less heat resistant than those of the parental strain. The mutants produce reduced amounts of dipicolinic acid—a spore-specific compound.

The heat resistance of the mutant spores can be partially restored by addition of gramicidin at the end of the growth phase (when sporulation begins) but not later. This result indicates that the mutant's behaviour is caused by lack of gramicidin and not by some pleiotropic change affecting both sporulation and antibiotic synthesis. If this interpretation is correct this is the first time that a direct relationship has been clearly shown between these two processes. It is noteworthy that although gramicidin is required early in sporulation its effect is exerted on a very late event. In its absence spores are still produced (albeit slightly defective) indicating that gramicidin is not essential for a major part of the developmental process.

It is possible that the insensitivity to gramicidin after sporulation has begun is associated with an inability to take up the compound. This type of behaviour has been correlated with the closure of the double prespore membrane, resulting in two membranes of opposite orientation (Cooney, Freese & Freese, *Spores* **VI**, 187 (American Society for Microbiology,

Washington, 1975)). At this stage active transport into the spore compartment can no longer take place so that perhaps exogenous gramicidin is unable to exert its effect.

The requirement for gramicidin at a specific time well before the acquisition of heat resistance suggests that the antibiotic has an indirect effect. More recent work from the same laboratory (Sarkhar, Langley & Paulus *Proc. natn. Acad. Sci. U.S.A.* **74**, 1478; 1977) indicates that gramicidin is a potent and specific inhibitor of transcription *in vitro*. This contrasts with the assumption made hitherto that the antibiotic increases the permeability of cell membranes (Sadoff *Spores* **V**, 157 (American Society for Microbiology, Washington, 1972)). This new work, which made use of a cell-free transcription system devoid of membrane, shows that gramicidin inhibits binding of RNA polymerase to DNA. The inhibition seems quite specific for certain regions of the DNA and this led the authors to suggest that the antibiotic specifically inhibits transcription of particular genes; perhaps those concerned with vegetative functions but not with sporulation. In all work of this kind, however, it is difficult to determine *in vivo* effects of a compound since observations made *in vitro* may be inapplicable to the cells' physiological conditions.

The studies of Paulus and coworkers show that gramicidin is unnecessary for the formation of functional spores though in its absence the spores have some altered properties. This is consistent with the idea that gramicidin acts at a time when it is possibly being produced for a quite different reason—as an antibiotic, for example. Such an interpretation is compatible with the



A hundred years ago

A WONDERFUL white aquamarine has been found in Perthshire which, when cut, has produced one of the most brilliant gems ever seen. It is said by many competent judges to be equal to her Majesty's celebrated Koh-i-noor, its refraction being very great both by day and night. It is of a pure pellucid liquid white, and is known as the Scotch Koh-i-noor. Its hardness is 8.0, and specific gravity 2.76. Mr. Bryce M. Wright, F.R.G.S., is its possessor.

From *Nature* **16**, 7 June, 237; 1877.

fact that other species of *Bacillus* produce different antibiotics (unrelated chemically and in their mode of action) and yet can still sporulate normally. Although gramicidin must be considered as peculiar to *B. brevis* its properties suggest modifying functions that may apply to antibiotics in other bacteria.

This latest work from Paulus's laboratory, and in particular the technique of mutagenesis, suggests useful methods for exploring the possible function of antibiotics in other strains of *Bacillus*. It may then be possible to decide, species by species, whether these compounds are products associated with prolongation of growth or have a role in the control of bacterial morphogenesis. □

Twenty years after

from P. Pitha

Some 150 scientists attended the Fifth Aharon Katzir-Katchalsky Conference held at the Weizmann Institute of Science, Rehovot, Israel on 2-6 May, 1977. The meeting, organised by the Israel Academy of Sciences and Humanities, was opened by the President of the State of Israel, Dr H. E. Ephraim Katzir, who chaired the first session. It was a welcome, although uncommon, sight to see a head of state participating in a scientific meeting, and could probably only happen in Israel, where the president has always been a scientist.

TWENTY years after its discovery by Isaacs and Lindenmann, how far has interferon fulfilled its clinical potential? Well-controlled clinical studies of the effect of interferon on infection with herpes zoster and hepatitis virus (T. C. Merigan, Stanford University) indicate that interferon treatment started after the onset of infection shortened the time that clinical symptoms of herpes zoster infection were apparent and suppressed virus production in patients chronically infected with hepatitis virus.

In all future trials, however, the supply of interferon may be the rate-limiting step, and a number of groups are actively developing large scale production methods for human interferon. Production of interferon by genetic engineering is also an attractive proposition and several groups have started work on what is undoubtedly

going to be a long and difficult project.

Another major handicap to clinical work has been the lack of purified interferon. Now E. Knight (Du Pont, Delaware) has purified human fibroblast interferon to homogeneity, obtaining a single polypeptide of molecular weight 20,000. However, the major clinical trials have used human leukocyte interferon which is a complex mixture containing distinct components separable by charge and size (W. Stewart, New York), none of them serologically identical with fibroblast interferon. Human lymphoblastoid interferon seems to be a mixture of both leukocyte and fibroblast interferon (J. Vilcek, New York). It is important to determine whether these differences are due to different primary structures, and hence different genes, or to differences in post-translational modification.

Several lines of evidence show that interferon is newly synthesised after induction, and not formed from an inactive precursor. Genetic analysis of human-mouse hybrids continues, although it is still not completely clear whether induction of human interferon requires two chromosomes or only one. Further characterisation of virus-resistant mutant 3T6 cells suggested that constitutive interferon production is involved in this resistance (C. Colby, New York).

It has been shown that interferon production is not regulated at the level of transcription by comparison of the mRNA levels (assayed by injection into *Xenopus* oocytes) with the amounts of interferon produced in human fibroblasts induced by poly (rI) poly (rC) in normal and superinduction conditions (in which high yields of interferon are produced) (P. Pitha, Johns Hopkins University).

P. Marcus (University of Connecticut, Storrs) reported that a VSV defective interfering particle produced a high yield of interferon at low multiplicity, possibly by formation of a double-stranded RNA within the cell (see *Nature* 266, 815; 1977). This is the best evidence so far that viruses and polynucleotides induce through a common mechanism—formation of double-stranded RNA. However, little is yet known about how the interferon gene(s) is depressed.

Interferon acts by first interacting with the cell membrane, an interaction involving both gangliosides and glycoproteins (H. Ankel, University of Wisconsin, Milwaukee; R. Friedman, National Institutes of Health, Bethesda). Sensitivity to interferon seems to be determined by the receptor located at the cell membrane, however, it is not yet clear whether the species specificity is determined at the same level (C. Chany, Paris; M. Revel, Weizmann

Institute, Rehovot). Work with cell-free systems has made it clear that interferon produces several different effects.

Thus it was shown that in cells of different species interferon treatment induces an increase in the phosphorylation of specific polypeptides. Revel found in interferon-treated cells, the increase of a specific protein kinase which inhibits translation and is cyclic AMP independent. Both the phosphorylation and the translational inhibition mediated by protein kinase are enhanced in the presence of double-stranded RNA. A heat stable, low molecular weight translational inhibitor (probably an analogue of oligo adenylic acid) was found in interferon-treated cells by I. M. Kerr (National Institute of Medical Research, London). For the synthesis of this inhibitor both double-stranded RNA and ATP are required. However, other cellular enzymes such as an endo RNase described in detail by P. Lengyel (Yale University) may be regulated by double-stranded RNA and ATP in interferon-treated cells. The latter two systems, however, were not dependent on the phosphorylation. However, there is as yet no explanation of why the translation of viral mRNA is preferentially inhibited in the intact cell.

In the past, investigation of interferon's antiviral effect has been primarily on the small lytic viruses, and W. Joklik (Duke University, North Carolina) continues his elegant studies on reovirus. However, attention has now shifted to the oncogenic viruses. E. Winocour (Rehovot) and Revel reported that when interferon is added to cells shortly after infection with SV40, it affects translation only. Three papers (Pitha; Friedman; A. Billiau, University of Louvain) on the effect of interferon on MuLV infection showed that in contrast to all other viral systems studied, a late event in virus replication was affected, probably a stage in virus maturation and assembly.

Amounts of purified human interferon similar to those required for the antiviral effect slow cell growth (Knight). Interferon also inhibits delayed type sensitivity, affecting both sensitisation and the expression of the sensitised state (E. De Maeyer, Paris). Interferon also increases the effects of lymphochoriomeningitis virus disease in the newborn mouse, although the mechanism is not known (I. Gresser, Paris). These reports all indicate that interferon may have functions other than its antiviral effect. An important distinction has emerged between type I interferon (virus induced) and type II (induced by antigens and mitogens), and E. Falcoff (Paris) reported that stimulation of B cells produces type I, and of T cells type II interferon. □

articles

Rb/Sr ages and geological setting of ancient dykes in the Sand River area, Limpopo Mobile Belt, South Africa*

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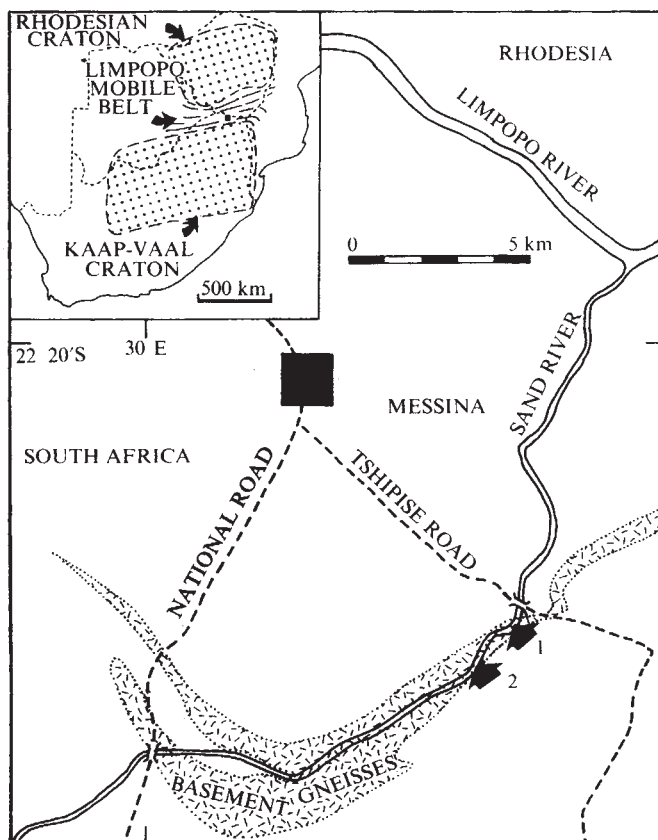
Rb/Sr whole-rock ages from two suites of tholeiitic dykes establish a pre-3,600 Myr history for some granodioritic gneisses in the Limpopo Mobile Belt and identify two previously unrecognised magmatic events in the evolution of this region. This has important implications with regard to relationships between the Belt and the adjacent Rhodesian and Kaapvaal Cratons.

WITHIN the Central Zone^{1,2} of the Limpopo Mobile Belt near Messina, South Africa, Bahnemann³ reported rocks which he considered to be basement to an overlying supracrustal sequence. A particularly spectacular exposure of both these units occurs in the valley of the Sand River (Fig. 1), where the basement rocks form a part of a structural unit, possibly a thrust and/or fold nappe, tightly infolded with the cover sequence. The presumed basement rocks, provisionally named the Sand River Gneisses, are comprised mainly of homogeneous peraluminous medium-grained quartz bearing grey gneiss and poorly banded peraluminous medium- to coarse-grained quartz bearing leucocratic gneiss. Detailed mapping (scale 1:100) at Locality 1 (Fig. 1) indicates that the basement gneisses occur as generally steep-dipping, tightly folded interlayered units showing small-scale interference patterns. In addition, both types of gneiss possess an early tectonic fabric and early quartzo-feldspathic dykes and veinlets that have been folded at least twice. Rb/Sr whole-rock analyses of both of these gneisses yield an isochron of 3858 ± 116 Myr (2σ) with an initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7012 ± 0.0002 (2σ) (B.R. and J.M.B., unpublished). The rocks regarded as being supracrustal to the basement gneisses are predominantly quartzite and iron formation interlayered with garnetiferous metapelatic rocks and with two-pyroxene gneiss, presumably a metavolcanic rock. A conclusive discordant relationship between the basement rocks and the supracrustal sequence has not yet been recognised, although preliminary structural fieldwork (R.E.P.F., in progress) is consistent with such an interpretation. In particular, this work suggests that the early tectonic fabric and early quartzo-feldspathic dykes found in the basement gneisses

are not present in the supracrustal rocks. No geochronological data are at present available for these supracrustal rocks.

In addition, as with similar strongly deformed and metamorphosed Archaean terrains in the Outer Hebrides⁴, West Greenland⁵ and Labrador⁶, the basement rocks contain deformed layers of amphibolite, which, in places are clearly discordant with respect to both the gneissic layering and

Fig. 1 Location map of the Sand River area showing the sampling sites. Shaded area denotes outcrops containing basement rocks.



*Contribution 9 of the South African International Geodynamics Project

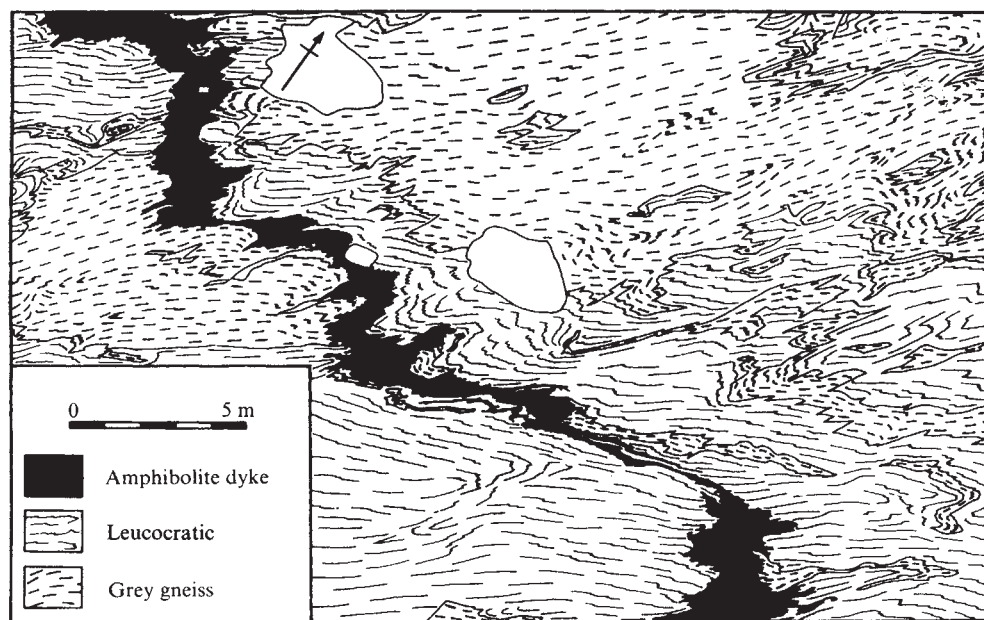
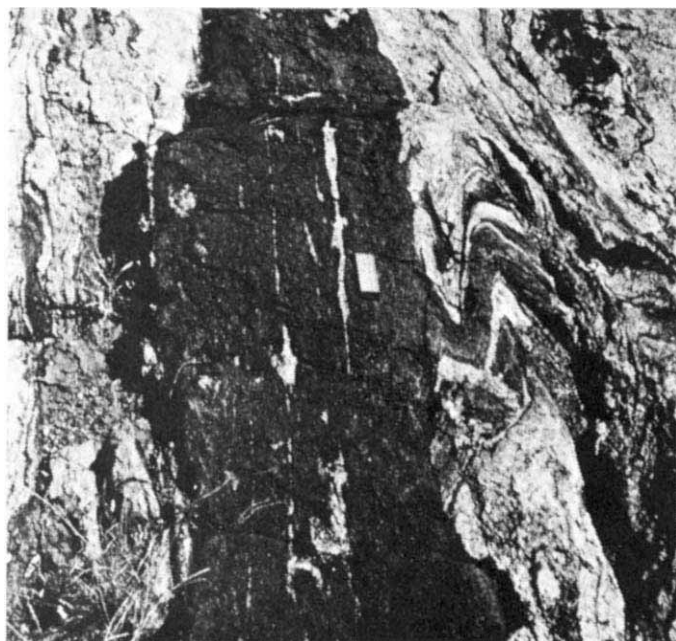


Fig. 2 Simplified geological map of a portion of the exposure of basement rocks in the Sand River area showing an older basement dyke. Blank areas are unexposed. Form lines represent pre-dyke fabric in the gneisses.

the mineral fabrics in the basement gneisses (Fig. 2) and are, therefore, interpreted as being basic dykes. These dykes post-date the early quartzo-feldspathic dykes and veinlets and have been folded and metamorphosed together with the gneisses in conditions of hornblende granulite facies. They have not, as yet, been recognised within the supracrustal rocks. At Locality 2 (Fig. 1), a suite of physically similar, deformed basic dykes show discordant relations with respect to both the grey and the leucocratic gneisses and to other, evidently older, basic dykes (Fig. 3). Therefore, at least two suites of strongly deformed amphibolite dykes are recognised in the basement rocks of the Sand River area, and these are not distinguishable in the field except where juxtaposed and where original discordant relationships are preserved.

Suites of samples of amphibolite, free of younger veinlets (Fig. 3), were carefully selected for Rb/Sr whole-rock iso-

Fig. 3 Photograph of the cross-cutting relationships between the older and younger dykes in the Sand River area. The scale resting on the younger dyke is 10 cm long. Note the deformed felsic veinlets in this dyke, and the large discordant angle between the two dykes.



topic analysis (Table 1) from both the older and younger dykes. These analyses yield isochrons⁹ of 3643 ± 102 Myr (2σ) with an initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7014 ± 0.0003 (2σ) for the older suite and of 3128 ± 81 Myr (2σ) for the younger suite (Fig. 4). No clear correlation exists between the K_2O concentrations and the Rb concentrations among these samples (Table 1) and on a plot of K_2O against Rb (Fig. 5), the K_2O concentrations are largely insensitive to changes in Rb concentration for samples from each dyke suite. It is likely, therefore, that each suite underwent some post-intrusive alteration of Rb concentrations. The isochrons, consequently, must reflect the time of such alteration. In view of the older Rb/Sr whole-rock isochron of the surrounding Sand River Gneisses, and the small initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of the dyke suites themselves, these alterations were probably deuteric in nature and involved the transfer of Rb between the wall rocks and the intruding magma. Such a process has been described by Fratta and Shaw¹⁰ and occurs before crystallisation of the dykes. The isochrons, therefore, very likely also closely approximate the time of intrusion of the dykes.

The initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the older dykes is greater than that normally postulated for the oceanic mantle ~ 3643 Myr ago¹¹⁻¹³. On the other hand, the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the younger dykes corresponds closely to the mantle value for ~ 3128 Myr ago. It is possible that the higher initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the older dykes is a consequence of crustal contamination and/or metasomatism ~ 3643 Myr ago. Such hybridisation would necessarily require either the addition of Sr with an $^{87}\text{Sr}/^{86}\text{Sr}$ ratio greater than ~ 0.7014 or the removal of Sr with an $^{87}\text{Sr}/^{86}\text{Sr}$ ratio less than ~ 0.7014 . These initial ratios, however, are also consistent with the possibility that these dykes were derived from distinct sources. If this is true, they support the suggestion by Condie¹³ that a heterogeneous mantle under continental areas formed early in Archaean times and also that a continental-type lithosphere of the type envisaged by Brooks *et al.*^{14,15} may have already been in existence in this region before ~ 3643 Myr ago.

The concept of a heterogeneous subcontinental mantle is further supported by major and trace element compositions of samples from these dykes. As far as major element chemistry is concerned, both generation of dykes are almost identical (Table 2). They have compositions of quartz-normative tholeiites showing significant iron and titanium enrichment, and they contain more iron and titanium than either the Ameralik dykes of West Greenland (13.30%

Table 1 Rb/Sr isotopic data for basic dykes

Sample	^{87}Rb (p.p.m.)	^{86}Sr (p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Sr}/^{86}\text{Sr}$	K_2O (weight %)	K/Rb
Older dykes						
B-75-57	2.64	40.8	0.0640	0.7047	0.85	742
B-76-25	1.76	11.7	0.1485	0.7090	0.93	1204
B-75-59	2.62	13.5	0.1921	0.7115	0.85	744
D2	2.97	11.0	0.2672	0.7151	0.97	770
D1	3.20	10.5	0.3013	0.7171	0.99	702
Younger dykes						
B-75-55	2.24	11.9	0.1861	0.7084	0.74	716
B-76-115	4.08	16.7	0.2416	0.7109	0.91	514
B-75-60	3.48	12.6	0.2724	0.7123	0.55	367
B-76-116	6.07	19.0	0.3150	0.7142	0.83	316
B-75-58	4.35	12.4	0.3464	0.7153	0.73	384
B-76-117	5.12	12.5	0.4058	0.7181	1.01	453
B-75-54	5.58	12.4	0.4461	0.7201	0.37	913

Twelve samples were collected for study. Of these, five samples were from demonstrably younger dykes, and four from the older dykes. The remaining three samples came from dykes which from a field standpoint, were of uncertain age. Powders of the samples were simultaneously spiked for Rb ($>99.2\%$ ^{87}Rb) and for Sr ($>99.3\%$ ^{86}Sr). ^{87}Rb and ^{86}Sr concentrations are precise to within 0.5% (1σ). The 1σ uncertainty in the $^{87}\text{Rb}/^{86}\text{Sr}$ ratio is $\pm 0.7\%$ while the 1σ uncertainty in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is $\pm 0.05\%$. Replicate analyses of the Eimer and Amend standard SrCO_3 yields 0.7080 ± 0.0001 (1σ). The data were tested by both the techniques of York⁷ and Brooks *et al.*⁸ to demonstrate that they scatter less than that amount predicted from experimental uncertainties alone. The ages and initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios were calculated by the technique of York⁹ using $1.39 \times 10^{-11} \text{ yr}^{-1}$ as the decay constant of ^{87}Rb . The K_2O concentrations were determined by X-ray fluorescence spectrometry and are precise to within 0.5% (1σ).

total Fe as Fe_2O_3 ; 0.86% (TiO_2)¹⁸ or the Säglekh dykes of Labrador (14.21% total Fe as Fe_2O_3 ; 1.20% TiO_2)¹⁷. Significantly, however, they have K_2O ($0.8\text{--}0.9\%$) contents greater than those characteristic of oceanic tholeiites ($<0.6\%$ K_2O)¹⁸, but consistent with the contents of K_2O in continental tholeiites (0.9% K_2O)^{19,20}. Furthermore, the older dykes have higher Sr concentrations than do the younger dykes, consistent with the possible existence of a heterogeneous source region.

The Limpopo Mobile Belt has previously been regarded as being largely of late Archaean–early Proterozoic age^{21,22}, and the emplacement of tholeiitic dykes at ~ 3643 Myr and 3128 Myr ago establishes two additional events in the crustal development of the region. Furthermore, the timing of these events supports the possibility that basement rocks to the Limpopo supracrustal rocks are exposed along the Sand River. Some of the supracrustal rocks near Messina are intruded by the Messina Layered Intrusion and are consequently believed to be older than the 3221 ± 48 Myr (2σ) Rb/Sr whole-rock isochron age of this body²³. Some of them are also presumed to be younger than the ~ 3643 Myr age reported here for the older dykes. Therefore, either the ~ 3128 -Myr old dykes should be found in the cover sequence, or the cover sequence exposed along the Sand River is younger than the cover sequence into which the Messina Layered Intrusion was emplaced. In either case, the deformation and high-grade metamorphism

which the rocks of the Limpopo Mobile Belt have undergone, must have occurred more recently than ~ 3128 Myr ago, and this deformation and metamorphism was not of sufficient intensity to obliterate the older isotopic ages.

With the exception of the rocks into which these dykes are intrusive, no other rocks have yet been reported in Southern Africa yielding Rb/Sr whole-rock ages as old as ~ 3643 Myr. Within the Kaapvaal and Rhodesian Cratons, however, granitic rocks yielding errorchrons of between ~ 3600 and ~ 3500 Myr have been reported^{24–26}, and major igneous and/or metamorphic activity occurred from ~ 3520 Myr ago²⁷ up to ~ 2700 Myr ago^{28–31}. Consequently, compared with the ages for the adjoining Rhodesian and Kaapvaal Cratons (Fig. 1), there is nothing particularly unique to the new ages recorded in the Limpopo Mobile Belt. It seems reasonable to assume, therefore, that the Limpopo Mobile Belt is floored by and otherwise contain rocks that are coeval, but not necessarily correlative with, those in the Rhodesian and/or Kaapvaal Craton.

The Lower Onverwacht komatiitic volcanic rocks in the Barberton greenstone belt were deposited sometime ~ 3500 Myr ago^{27,29,32,33} within the span of time from ~ 3643 to ~ 3221 Myr ago during which the Limpopo supracrustal

Fig. 4 Isochron plot of the Rb–Sr isotopic data from the basement dykes in the Sand River area.

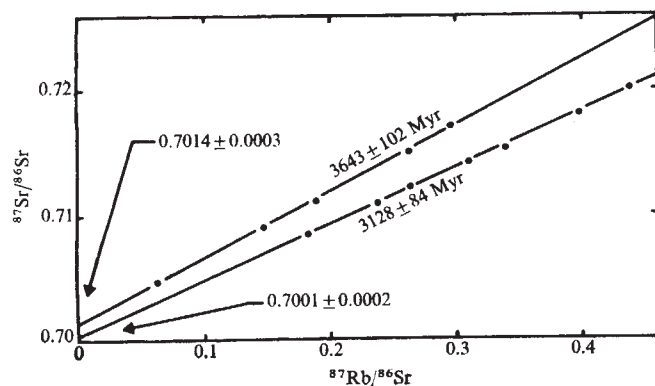
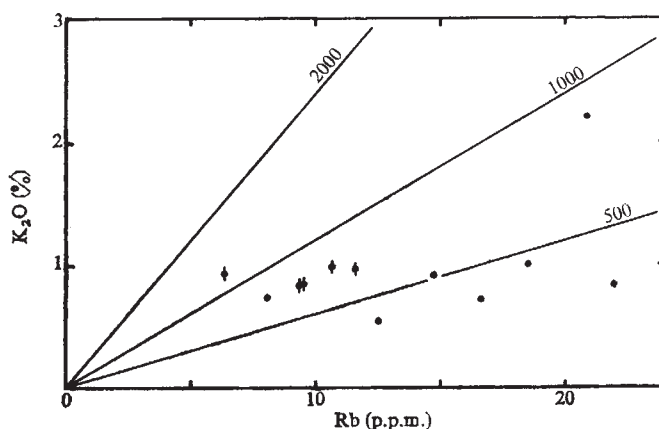
Fig. 5 Plot of K_2O (weight %) against Rb (p.p.m.) for the dyke samples analysed in this study. Lines of equal K/Rb ratios are shown for comparison. ●, Older dyke suite; ■, younger dyke suite.

Table 2 Average chemical compositions of the basic dykes

	Older dykes*	Younger dykes†
SiO ₂	49.78±2.63	49.29±2.74
Al ₂ O ₃	12.77±0.44	12.69±0.40
TiO ₂	2.48±0.53	1.99±0.52
Fe as Fe ₂ O ₃	16.97±3.69	16.49±1.72
MnO	0.22±0.06	0.20±0.03
MgO	5.12±1.53	5.68±1.10
CaO	8.27±0.52	9.05±0.94
Na ₂ O	2.37±0.35	2.30±0.18
K ₂ O	0.91±0.07	0.84±0.20
P ₂ O ₅	0.44±0.10	0.27±0.11
H ₂ O-	0.10±0.05	0.23±0.15
L.O.I.‡	0.68±0.14	1.03±0.12
Rb (p.p.m.)	9.50	15.9
Sr (p.p.m.)	182.0	144.4
K/Rb	795.0	439.0
Rb/Sr	0.0521	0.110
C.I.P.W. norm§		
QZ	4.74	2.85
OR	5.38	4.96
PLAG	41.57	41.28
(% An)	(52)	(52)
CPX	13.87	17.71
OPX	21.77	21.49
AP	1.04	.64
MT	4.92	4.78
IL	4.71	3.78
Normative specific gravity	3.13	3.13

Major element concentrations were determined by X-ray fluorescence spectrometry. Trace element concentrations determined by isotope dilution mass spectrometry. The scatter in the analyses are expressed as single standard deviations.

*Average of 5 samples.

†Average of 7 samples.

‡Loss on ignition.

§Calculated assuming 80% of total Fe occurs as ferrous iron.

rocks were presumably deposited. Although no compelling field evidence for a sialic basement to the Barberton greenstone belt has yet been reported, petrogenetic considerations for the evolution of komatiitic magmas suggest that a continental environment existed²⁴. Furthermore, we and others (T. N. Clifford and C. Guerin, personal communication) have observed, on the south-western flank of the Barberton greenstone belt, outcrops of sialic gneisses containing strongly deformed basic dykes. These outcrops are

very similar in aspect to those of the Sand River area, and it now seems likely that detailed structural and isotopic studies, of the type reported in this paper, in the Barberton and Swaziland areas and in particular in the so-called migmatitic-gneiss complex terrains, will confirm the presence of ancient pre-greenstone belt sialic crust²⁵.

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Is an early calcium flux necessary to stimulate lymphocytes?

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Concentrations of concanavalin A or the calcium ionophore A23187 that are optimal for the transformation of pig or mouse lymphocytes do not normally cause a measurable increase in calcium influx compared with unstimulated cells. If the cells are treated with the mitogens in conditions where a measurable increase in calcium influx occurs, no stimulation of the cells can occur while the flux is maintained. If any early influx of extracellular calcium is necessary for stimulation, then a much smaller increase in the total concentration of cellular calcium than reported previously is sufficient to allow the entry of lymphocytes into the cell cycle.

SEVERAL reports have indicated that mitogens which stimulate the transformation of T lymphocytes cause an increased rate of calcium influx within 1 h (refs 1–6). The magnitude and duration of the calcium fluxes reported for both resting and stimulated lymphocytes, however, vary widely. Thus, Freedman *et al.*⁶ have described a gated calcium flux into mouse T cells stimulated by optimal concentrations of concanavalin (con A) in which a large influx is completed within one minute of the addition of the lectin, after which the gate shuts and the subsequent uptake of calcium is the same as in unstimulated cells. In contrast, Whitney and Sutherland³ have found that there was a very large and continuous increase in Ca²⁺ uptake

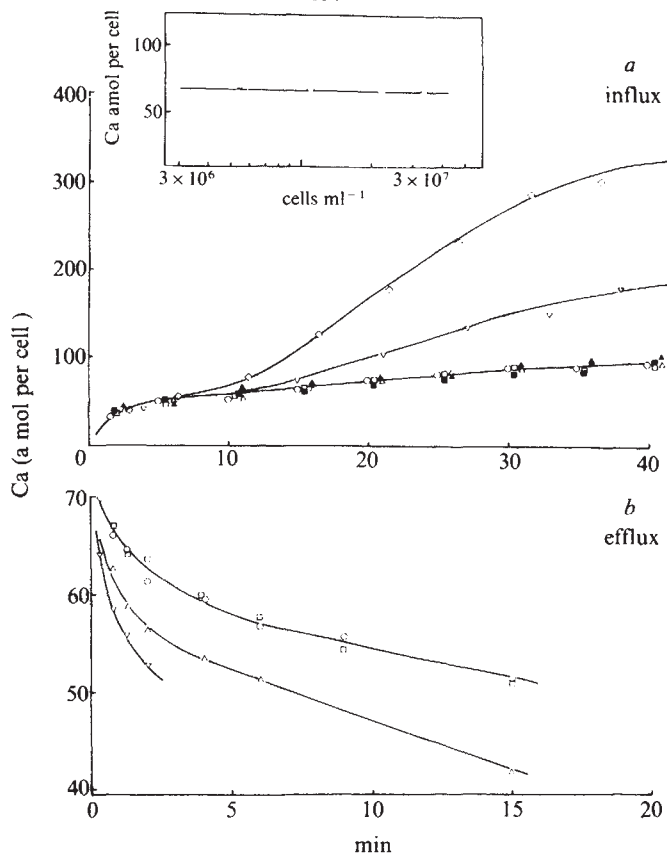


Fig. 1 *a*, Uptake of Ca^{2+} into unwashed pig lymphocytes (10^7 cells ml^{-1}) without FCS at $37^\circ C$, at increasing concentrations of ionophore. ○, Control; □, 0.076 μM ; △, 0.19 μM ; ▽, 0.95 μM ; ◇, 1.9 μM . Solid symbols indicate Ca^{2+} taken up by cells pre-incubated with ionophore for 1 h at $37^\circ C$ before addition of $^{45}Ca^{2+}$. ■, 0.076 μM ; ▲, 0.19 μM . $^{45}CaCl_2$ (0.88 μmol Ca^{2+} mCi^{-1} ; Amersham) in aqueous solution was added to culture medium to give a final activity of 6 μCi ml^{-1} . This solution was filtered through a 0.22 μm Millipore filter to remove particulate matter, and aliquots were dispensed into cleaned plastic vials and incubated at $37^\circ C$. Filtering the incubation medium improved the reproducibility of calcium uptake levels between different cell preparations. A stock solution of A23187 (19 mM in DMSO) or con A (1 mg ml^{-1} ; Miles-Yeda) was diluted into filtered medium and added to the incubation followed by 50 μl of stock cell suspension to give a final concentration of 10^7 cells ml^{-1} in a total volume of 1.0 ml. Incubations were made under 5% CO_2 in air, with or without continuous stirring in each system¹². Samples of 200 μl were withdrawn and diluted into 10 ml of wash buffer (either 146 mM NaCl, 10 mM Tris, 0.4 mM Ca-EGTA, pH 7.2; or culture medium) at $3^\circ C$ and immediately filtered through 2.5-cm Whatman GF/C filters. The cells were further washed with 30 ml of the same wash solution at $3^\circ C$ within a total dilution and wash time of 4 s, and the filters dried and counted for radioactivity. A wide range of variations in this protocol were examined. The $^{45}Ca^{2+}$ retained was decreased by using larger wash volumes up to 60 ml (<10%); by lengthening the wash time to 45 s at $3^\circ C$ (<2.5%); and by raising the temperature of the wash medium to $37^\circ C$ (<45%), whereas removal of EGTA and the addition of 5 mM Ca^{2+} to the wash medium increased the $^{45}Ca^{2+}$ retained by up to 35%. The viability of the cells was unaffected by the filtration and washing procedures, as judged by their retention of $^{51}Cr^{3+}$ and their subsequent transformation by con A. We conclude that the calcium levels measured by the basic protocol represent most or all of the $^{45}Ca^{2+}$ that has been taken up or tightly bound to the cells during incubation at $37^\circ C$. In experiments in which transformation of cells from the same preparation was followed after the flux measurements, $^{45}Ca^{2+}$ uptake was measured at the optimal cell concentration for transformation of 3×10^6 ml^{-1} . Aliquots of the cells from each incubation for $^{45}Ca^{2+}$ uptake were subsequently shown to be transformed to the same extent as control samples of the same cell preparation set up for transformation (see Fig. 2). *b*, Efflux of Ca^{2+} from unwashed pig lymphocytes loaded with $^{45}Ca^{2+}$ for 30 min at $37^\circ C$ as for uptake measurements, and concentrated by centrifugation for 30 s at 14,000*g* (viability unaffected) before diluting into culture medium, with or without ionophore present, to give a final concentration of 10^7 cells ml^{-1} at $37^\circ C$. $^{45}Ca^{2+}$ retained in the preloaded cells was measured as for the uptake experiments. ○, Control; □, 0.19 μM ; △, 0.38 μM ; ▽, 1.15 μM .

for at least 24 h in cells stimulated with con A. Since the timing and magnitude of the early events after stimulation are critical in defining the triggering mechanism, and even a requirement for Ca^{2+} in the culture medium during the early stages of stimulation has been questioned⁷⁻¹⁰, we have attempted to determine whether either a gated pulse or a prolonged influx of calcium is obligatory for transformation. We have found that in conditions in which the mitogens cause a measurable increase in calcium influx in pig and mouse lymphocytes, the cells cannot become committed to transform while the influx is maintained. The limit to any calcium influx during the commitment of the cells to subsequent transformation corresponds to an increase in the total cellular concentration of less than 50 μM Ca^{2+} .

Calcium fluxes and transformation stimulated by A23187 and con A

Lymphocytes from pig mesenteric lymph node, or BALB/c mouse spleen were teased into culture medium containing RPMI 1640 (0.43 mM Ca^{2+}), supplemented with 24 mM sodium bicarbonate; 20 mM Tris-HCl; and gentamicin (10 μg ml^{-1}), pH 7.2. These unwashed cells were either used without further treatment, or the cells were washed by centrifugation on to 6.0% Ficoll and 16.7% Triosil, and the interfacial layer of cells centrifuged and resuspended twice in culture medium. For some Ca^{2+} uptake and transformation experiments (see Figs 1 and 2 for methods), 10% heat-inactivated foetal calf serum (FCS; Flow Labs) was also added to the culture medium. The FCS had little effect on DNA synthesis in transformed cells (for example compare Fig. 2*a* and *b*).

Calcium influx into unwashed pig lymphocytes in culture medium measured at 30 min in the absence of ionophore was found to be almost constant at 70–80 amol per cell, over the range of cell concentrations from 3×10^6 to 3×10^7 cells ml^{-1} (Fig. 1*a*, inset). This corresponds to an average cellular calcium concentration of about 0.6–0.7 mM, assuming a cell volume of 120 μm^3 (ref. 11), compared with an extracellular concentration of 0.43 mM. Calcium uptake into the cells at increasing concentrations of ionophore is shown in Fig. 1*a*. It can be seen that no significant stimulation of calcium uptake can be detected at ionophore concentrations less than 0.3 μM , but increasing the concentration further causes a progressive increase in $^{45}Ca^{2+}$ retained by the cells. Similar calcium uptake experiments were also performed with cells that had been pre-incubated for 1 h at $37^\circ C$ with ionophore to ensure equilibration before adding $^{45}Ca^{2+}$. The uptake of $^{45}Ca^{2+}$ is unaltered (Fig. 1*a*), indicating that the absence of a detectable increase in calcium flux below 0.3 μM is not due to slow equilibration of the ionophore with the cells. Ionophore concentrations less than 0.3 μM that have no detectable effect on calcium uptake also have no effect on the efflux profiles from cells preloaded for 30 min with $^{45}Ca^{2+}$ without ionophore present (Fig. 1*b*). We can conclude that any change in the average cellular calcium concentration is too small to detect against the fluxes occurring in the control cells.

The transformation of the same cell preparations used to measure calcium uptake is shown in Fig. 2*a*. It is clear that a measurable increase in $^{45}Ca^{2+}$ uptake at 30 min only occurs at concentrations of ionophore that abolish transformation measured as DNA synthesis at 42–48 h. A very similar relationship between calcium uptake and transformation is observed in the presence of FCS (Fig. 2*b*). An increase in ionophore concentration of about tenfold is required for transformation, presumably because the serum binds a major portion of the ionophore. At the same time, the calcium uptake profile is shifted to higher ionophore concentrations, so that no increase in calcium uptake

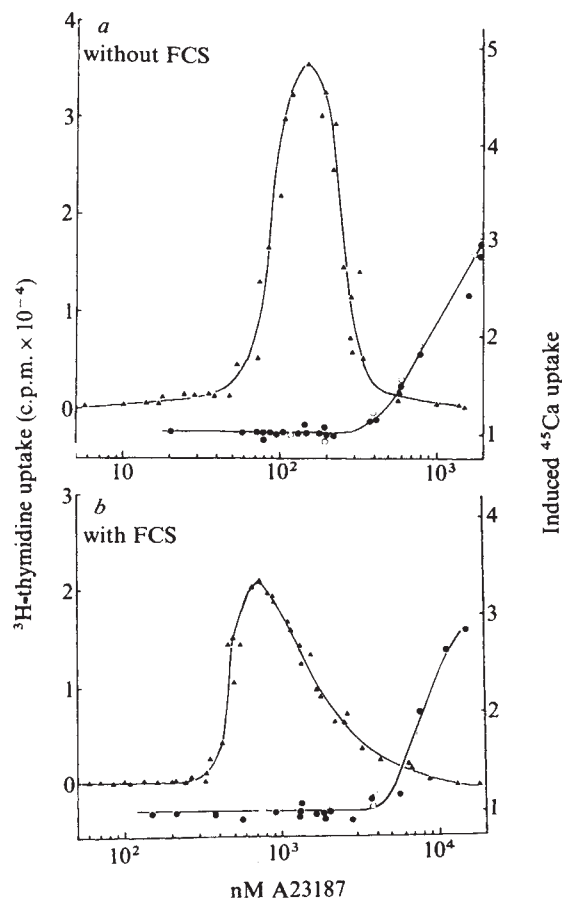


Fig. 2 a, Uptake of $^{45}\text{Ca}^{2+}$ into unwashed pig lymphocytes in culture medium without FCS after 30 min at 37°C as a function of ionophore concentration, expressed as a ratio to uptake in cells without ionophore. The uptake ratios were the same at 10^7 cells ml^{-1} ($\bullet$) and at 3×10^6 cells ml^{-1} ($\circ$), which was the optimal concentration for transformation measured as DNA synthesis ($\blacktriangle$) in the same cell preparation and medium. Aliquots (200 μl) of the cell suspension at 3×10^6 cells ml^{-1} were incubated with ionophore for 42 h in round-bottomed Cooke Microtitre plates under 5% CO_2 in air, before addition of 0.3 μCi ^3H -thymidine to each well. The cells were collected with a multiple harvester, washed with water and counted for radioactivity. **b**, Same data as in **a** with 10% FCS added to the medium.

is observed at ionophore concentrations that cause transformation.

In the transformation experiments with ionophore, the levels of DNA synthesis were very similar to those obtained with optimal concentrations of con A (Figs 2a and 3a). Removal of the ionophore after 24 h by centrifugation and washing the cells in fresh medium, approximately doubles the levels of DNA synthesis measured between 42 and 48 h. The highest proportion of pig cells responding to A23187 or con A was approximately 70%, as judged by a count of blast cells at 42 h, similar to the response to ionophore first described by Maino *et al.*¹²

The pattern of response to con A for calcium fluxes and transformation in pig and mouse lymphocytes is shown in Fig. 3a and b. For pig cells, no significant increase in calcium flux was observed at concentrations of con A between 0.1 and $100 \mu\text{g ml}^{-1}$. The average calcium uptake ratio at con A concentrations that cause significant transformation was 1.04 (s.d. of 0.04 in 26 experiments). The corresponding data for mouse cells shows a significant increase in calcium influx above about $5 \mu\text{g ml}^{-1}$ con A, where transformation is almost completely inhibited. Thus, although the calcium uptake data for con-A-stimulated cells is more variable than the corresponding ionophore

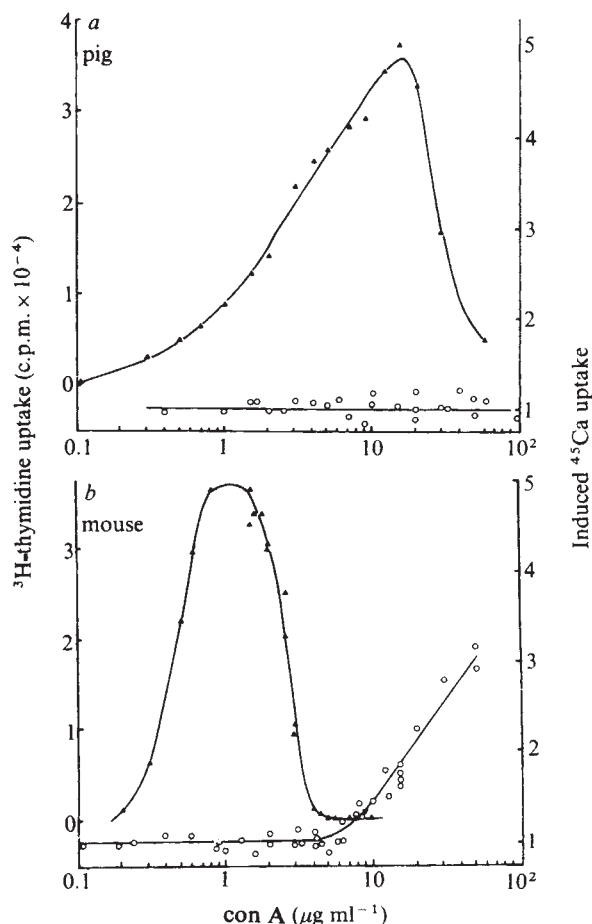
data, for both mitogens at optimal concentrations for transformation there is no significant increase in calcium influx at 30 min.

Commitment to transformation can occur after but not during an early calcium influx

The experiments described strongly suggest that an immediate calcium influx large enough to be measured against the influx into resting cells is not required for transformation, but is apparently inhibitory. We therefore attempted to induce large calcium influxes into the cells to determine whether they would remain viable, and in what conditions they would subsequently transform. Large increases in $^{45}\text{Ca}^{2+}$ uptake at 30 min were induced in pig cells by high concentrations of ionophore (Fig. 1a). If the ionophore was then removed by washing, and the cells resuspended in optimal concentrations of ionophore, the viability was unaltered and the cells subsequently transformed normally. Mouse lymphocytes were rapidly killed at ionophore concentrations optimal for pig cell transformation (40% viability after 3 h in $0.3 \mu\text{M}$ A23187) and only small stimulations of DNA synthesis corresponding to about 15% of the maximal stimulation of con A were observed.

In contrast to unwashed pig lymphocytes, the $^{45}\text{Ca}^{2+}$

Fig. 3 a, Effect of con A on the uptake of $^{45}\text{Ca}^{2+}$ into pig lymphocytes at 3×10^6 cells ml^{-1} ($\circ$) in culture medium without FCS after 30 min at 37°C expressed as a ratio to uptake cells without con A, and compared with DNA synthesis in the same cell preparation at 42–48 h ($\blacktriangle$). In experiments with con A, both $^{45}\text{Ca}^{2+}$ uptake and transformation were unaffected by washing the cells. **b**, Same data as in **a** for mouse lymphocytes.



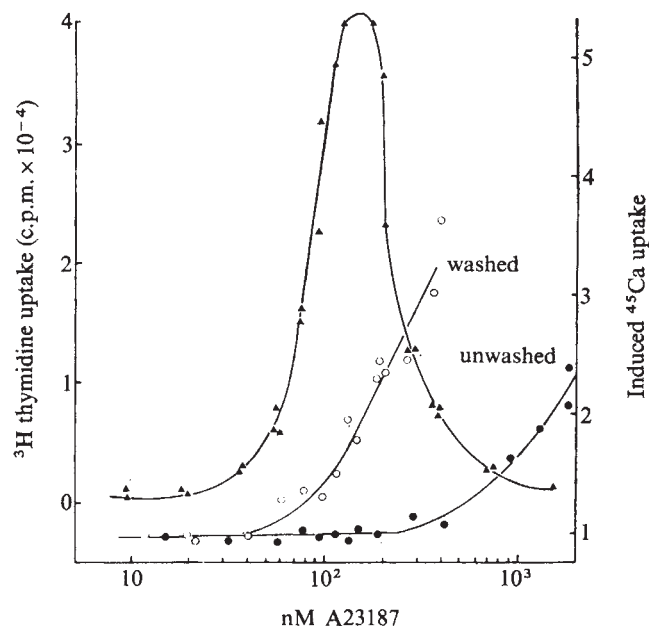


Fig. 4 Uptake of $^{45}\text{Ca}^{2+}$ into pig lymphocytes (3×10^6 cells ml^{-1}) either unwashed (●) or washed twice (○) (see text). DNA synthesis at 42–48 h (▲) in the same cell preparation was unaffected by washing the cells.

uptake profile was shifted to lower ionophore concentrations after washing the cells as described previously (Fig. 4). This treatment had no effect on the extent of transformation or the optimal concentration of ionophore required, so that the washed cells showed a significant increase in calcium uptake after 30 min at the ionophore concentrations optimal for transformation. When the time course of calcium uptake was followed by addition of $^{45}\text{Ca}^{2+}$ at increasing intervals after the addition of ionophore and comparing the uptake of $^{45}\text{Ca}^{2+}$ in cells with and without ionophore 30 min later, it was found that the initial increase in $^{45}\text{Ca}^{2+}$ uptake due to the ionophore decreased to the same level as in the control cells after about 4 h (Fig. 5). Subsequently the $^{45}\text{Ca}^{2+}$ uptake was indistinguishable from the control cells for at least 20 h, when the calcium uptake ratio was 1.02 (not shown). By analogy with the effect of FCS on the calcium uptake profile (Fig. 1a and b), it is likely that the large influx of $^{45}\text{Ca}^{2+}$ occurs when any extracellular material from the lymph tissue capable of buffering the ionophore has been depleted by washing, so that more of the ionophore interacts immediately with the cells than in the unwashed preparations. The subsequent decrease in $^{45}\text{Ca}^{2+}$ may result from redistribution, or even metabolism, of the ionophore in the cells. If the increase in $^{45}\text{Ca}^{2+}$ uptake is maintained by further additions of ionophore, no transformation occurs. On the other hand, if the optimal concentration of ionophore is added to the washed cells in ten equal aliquots at 30-min intervals, no significant increase in $^{45}\text{Ca}^{2+}$ uptake is observed, although the cells transform normally as if the ionophore had been added as a single dose.

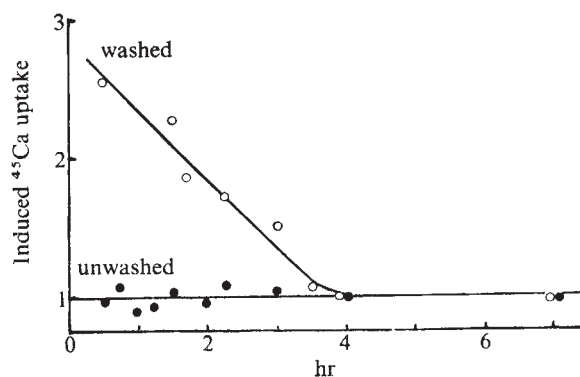
Calcium fluxes and transformation

Contrary to previous studies, the present results consistently demonstrate that a persistent increase in calcium influx large enough to be detected against the normal levels of calcium exchange in unstimulated lymphocytes is sufficient to abolish the subsequent transformation of the cells. We cannot account comprehensively for this discrepancy, although we would emphasise that to demonstrate the relationship between the calcium flux profile and

transformation (for example, in Figs 2, 3 and 4), it is essential that the measurements are carried out on a single cell preparation using the same mitogen solutions and at optimal cell concentrations for transformation. Small differences between cell preparations and larger differences between batches of mitogens were sufficient to completely obscure the pattern of results. This is particularly important in comparing calcium fluxes with transformations caused by short exposures (for example, 1–5 h) to high ionophore concentrations for which the optimal concentration is significantly higher than for long term stimulations (T.R.H. *et al.*, unpublished). The importance of the experimental conditions is also demonstrated by the facility with which apparently conflicting results can be generated by small changes in the treatment of the cells, where washing pig lymphocytes can induce a significant increase in calcium influx at optimal ionophore concentrations similar to the fluxes described previously. The stimulated influx, however, is transient, and the cells can only become committed to transform after the calcium influx has returned to the unstimulated level.

In most of the previous reports^{1–6,8–10} the increase in Ca^{2+} influx due to ionophore, con A or phytohaemagglutinin can be calculated as ranging between 60 amol per cell and 35×10^3 amol per cell, which if retained in the cell would correspond to an increase in total cellular calcium concentration of 0.5–300 mM. If these large fluxes occur in cells that are to transform, we assume that nearly all of the calcium would have to be pumped out of the cell again or be sequestered in organelles, since 1 mM calcium in the cytoplasm would be sufficient to inhibit glycolysis and protein synthesis, which are both stimulated in transformed cells^{13,14}. It is likely that at supraoptimal concentrations of ionophore, pig lymphocytes are unable to cope with the large and continuous calcium influxes from the medium (0.43 mM Ca^{2+}) and that the cytoplasmic concentration therefore increases to a level which prevents the generation of a mitogenic signal and is eventually cytotoxic. The only report in which the experiments carry the implication that the cell can successfully remove a large calcium influx from the cytoplasm, is the gated calcium pulse described by Freedman *et al.*^{6,9,10}. The kinetic implications of the pulse, which corresponded to an increase in the total cellular concentration of about 2 mM, are important in considering it as a possible mitogenic signal. When the con A and $^{45}\text{Ca}^{2+}$ were added to the mouse lymphocytes at the same time, the increase in the $^{45}\text{Ca}^{2+}$ uptake ratio remained constant after the first minute for at least 20 h (Freedman, M. H., personal communication),

Fig. 5 Uptake of $^{45}\text{Ca}^{2+}$ into unwashed (●) and twice-washed (○) pig lymphocytes, as a function of time. The optimal ionophore concentration for transformation (0.10 μM) was added at start, and $^{45}\text{Ca}^{2+}$ added after increasing time intervals. After 30 min incubation with $^{45}\text{Ca}^{2+}$, the uptake was measured and is expressed as a ratio to uptake in control cells.



whereas the uptake of $^{45}\text{Ca}^{2+}$ added 5 min after the con A, when the gate was shut, was the same as in control cells.

Systematic examination of kinetic models for the calcium fluxes after the gate has shut, in which we have assumed that the calcium entering the cell during the pulse remains accessible for subsequent exchange with the extracellular calcium, has indicated that all of the models require the $^{45}\text{Ca}^{2+}$ uptake ratio to drop from a maximum value after the gate shuts, towards the level of unstimulated cells. Since this is contrary to what has been observed experimentally, we conclude that the gated calcium pulse into the stimulated cells must be deposited immediately at intracellular sites where it is inaccessible for exchange. The subsequent exchange fluxes are then unaffected by the pulse and are the same as in unstimulated cells.

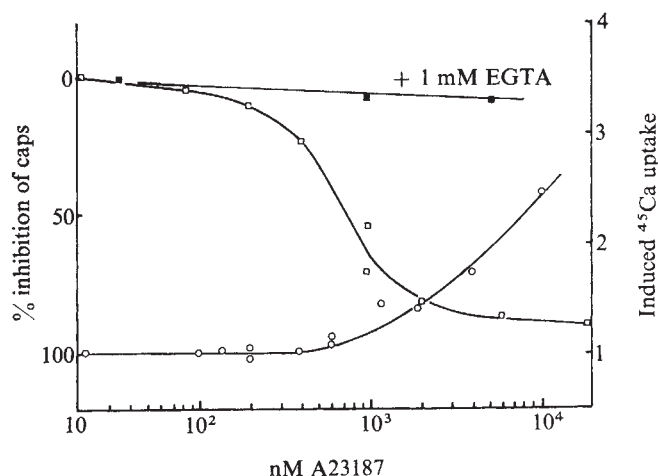


Fig. 6 Inhibition by A23187 of capping (± 1 mM EGTA ■, □) by fluorescein-labelled con A (FITC-con A) on washed pig lymphocytes, compared with the calcium uptake ratio at 30 min for cells from the same preparation (○). Cells at $3 \times 10^6 \text{ ml}^{-1}$ were incubated in culture medium with ionophore for 20 min at 37°C ; FITC-con A ($200 \mu\text{g ml}^{-1}$) was added, and the cells fixed in 1% paraformaldehyde after a further 10 min at 37°C before examination by fluorescence microscopy. Caps, defined as fluorescent staining restricted to less than half of the cell surface, were present on $30 \pm 5\%$ of the cells, and the results are expressed as a percentage of the number of cells capping in the presence of ionophore. Similar inhibition of capping by the ionophore, was observed with fluorescein-labelled antibody to immunoglobulin which forms caps on more than 90% of the B cells in the absence of ionophore (not shown).

The important features of the gated pulse are that, although it is large, the calcium does not remain free in the cytoplasm where it would be accessible for exchange, and that after the gate shuts, the cell does not have to clear from the cytoplasm a continuous large influx of calcium. Thus the cytoplasmic functions essential for transformation would not be inhibited. Since the pulse is largest at optimal con A concentrations for transformation, is observed in T or B cells only when stimulated with the appropriate mitogens¹⁰, and is modulated by cyclic nucleotides⁶, it has many of the characteristics expected for a mitogenic signal. On the other hand, the absence of a detectable calcium pulse in the present work is unlikely to be due to any insensitivity in the technique used, because the calcium uptake levels in unstimulated cells are similar to those observed by the centrifugation methods, and reproducible increases in the calcium fluxes can be measured at supraoptimal ionophore and con A concentrations that show striking internal consistencies in the transformation data.

If we accept that in some experimental conditions the

cells can transform without requiring an increase in calcium influx that can be measured against the large exchange fluxes occurring in resting lymphocytes, we can set a limit to any increase in the total cell calcium concentration that is obligatory for transformation. For pig lymphocytes at optimal ionophore concentrations, the calcium influx ratio is 1.01 ± 0.06 (33 experiments with FCS) and 1.00 ± 0.08 (22 experiments without FCS). This is equivalent to a change in calcium flux of less than $\pm 6 \text{ amol calcium per cell}$, or a change in the total cellular concentration of less than $50 \mu\text{M}$ calcium. Similar conclusions apply to any induced calcium influx in pig or mouse lymphocytes stimulated by optimal con A concentration. These results raise the question of whether the ionophore or con A act through a calcium flux, and whether calcium is even required in the extracellular medium during the early stages of stimulation, which is at present unclear⁷⁻¹⁰.

There is a further complication in that there is compelling evidence that at least some T-cell subpopulations require macrophages for transformation¹⁵, and it has also been suggested that the ionophore stimulates lymphocytes indirectly by causing the release of soluble factors from macrophages. Although macrophages were not removed from the cell preparations used in the present studies, there is little doubt that the calcium fluxes at supraoptimal ionophore or con A concentrations were too large to be occurring in a small population of cells which were not lymphocytes. We therefore looked for evidence of a direct and immediate action of the ionophore on the plasma membrane of pig lymphocytes, and found that the ionophore inhibits cap formation by fluorescein-labelled con A, at concentrations at which an increased calcium flux can be detected (Fig. 6). This is similar to the observation by Schreiner and Unanue¹⁶ that the ionophore inhibits cap formation by antibody to the immunoglobulin receptor on mouse B lymphocytes, and strongly suggests that the ionophore is also interacting directly with the lymphocytes in the concentration range which causes transformation. Since the inhibition of cap formation requires calcium in the medium (Fig. 6), but not magnesium, we think it is very probable that the ionophore must cause an influx of calcium at optimal concentrations for transformation that is too small to measure by the present technique against the large fluxes in resting cells. The significance of such a small flux as a mitogenic signal and the requirement of extracellular calcium for the early stages of stimulation remain to be determined. The present work does not exclude the possibility that a small increase in cytoplasmic calcium concentration, within the limits described, is a component of the signal that commits the cells to transform.

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Endogenous opioid peptides: multiple agonists and receptors

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Opioid peptides were assayed by inhibition of ^3H -naloxone and ^3H -leu-enkephalin binding in brain homogenates and by depression of contractions of the guinea pig ileum and mouse vas deferens. We conclude that the opioid peptidergic system has agonists of different characteristics which interact with more than one type of receptor.

SOON after the publication¹ of the structures of Met-enkephalin and Leu-enkephalin and the fact that Met-enkephalin is present in β -lipotropin as the sequence 61–65, many papers have appeared which showed that peptides starting with the tyrosine residue 61 of β -lipotropin and having different lengths of amino acid residues interact with the opiate receptors in brain homogenates, guinea pig ileum and mouse vas deferens^{2–7}. The pharmacological properties of these peptides are sufficiently different that the question arises of whether or not they interact with identical receptor populations^{8,9}. Evidence for the presence of more than one species of opiate receptor may be of considerable importance in the analysis of the respective roles of the short- and long-chain opioid peptides. Related to this problem are recent findings which have made it doubtful that the many synthetic and semisynthetic surrogates of morphine in the morphine, morphinan, benzomorphan and phenylpiperidine series of compounds interact with a homogeneous group of receptors^{10–14}. Martin and his colleagues^{12,13} in particular have postulated from behavioural and neurophysiological observations on the chronic spinal dog that there are at least three different opiate receptors: typically morphine-like drugs interact with the μ -receptor whereas certain benzomorphans which do not substitute for morphine in the morphine-dependent monkey but are potent anti-nociceptive agents, for example ketocyclazocine and ethylketocyclazocine, interact with the κ -receptor. In addition, they distinguish the σ -receptor, for which the prototype is N-allylnorcyclazocine.

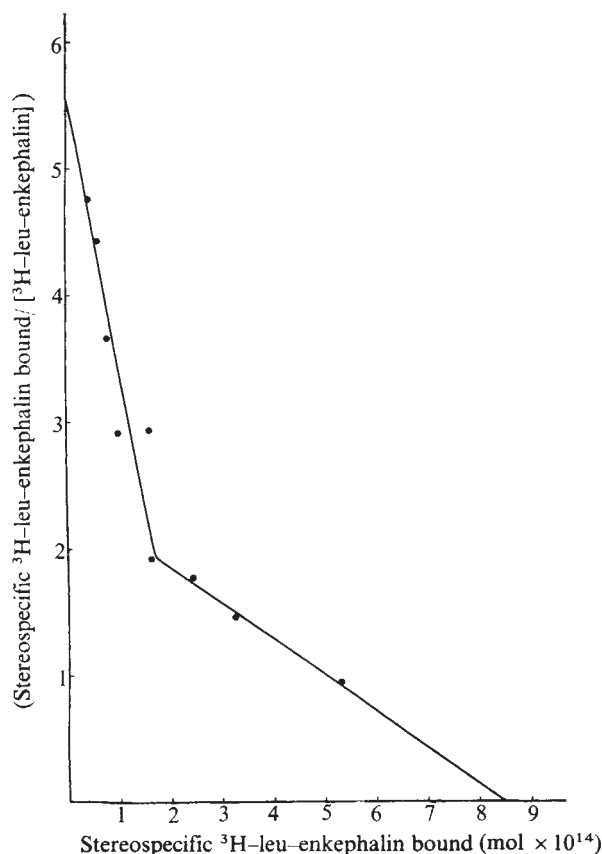
Differentiation of opioid receptors

Our aim was an analysis of this problem at the cellular level. Receptors of different binding properties present in a mixed receptor population are readily differentiated when antagonists are used which are specific for the different receptors. If such specific antagonists are not available, the agonist activities are assayed simultaneously in several systems. If the rank order of potency varies in parallel in the different assays, then the assay tissues may be assumed to have identical receptors. If, on the other hand, there is no correlation of the rank order of potency in the bio-assay systems, the receptor populations are not identical¹⁵.

We used two assays based on pharmacological responses—the depression of the electrically-induced contractions of the guinea pig ileum or its myenteric plexus–longitudinal muscle preparation^{16,17} and of the mouse vas deferens¹⁸. The other two systems are based on the inhibition of binding of labelled ligands in brain homogenates^{19–21}. The primary ligands were ^3H -naloxone (4 Ci mmol^{-1} , 1 nM ;

National Institute on Drug Abuse) and ^3H -leu-enkephalin (45.6 Ci mmol^{-1} , 0.86 nM ; Radiochemical Centre, Amersham). The technique used has already been described⁸; the incubation of the homogenates of guinea pig brain (19 mg per 2 ml Tris buffer pH 7.4 at 0°C , without Na^+) was carried out at 0°C for 150 min to reduce enzymatic inactivation to its minimum. In these conditions maximum specific binding of either ligand was reached after 120 min. Specific binding was the difference between the values obtained in the absence and presence of 50 nM of the potent antagonist (–)-2-(3-furymethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan (Mr 2266 from Dr H. Merz, C. H. Boehringer)²². At the same concentration, the (+) isomer had no significant inhibitory effect on binding. The specific binding was 50–60% of the total binding. There are apparently two binding sites for ^3H -leu-enkephalin (Fig. 1), $18.8 \pm 1.7\%$ of which have a K_D of $0.47 \pm 0.09\text{ nM}$ and $81.2 \pm 1.7\%$ a K_D of $4.98 \pm 1.15\text{ nM}$ ($n=4$). Inhibition with cold Leu-enkephalin gave an ID_{50} (concentration for 50% inhibition) of $3.55 \pm 0.37\text{ nM}$ ($n=18$), a value close to that of the K_D of the binding site of lower affinity. The

Fig. 1 Specific binding of ^3H -leu-enkephalin in a homogenate of guinea pig brain, incubated at 0°C for 150 min. Abscissa, bound ^3H -leu-enkephalin (mol^{14} per 19 mg brain); ordinate, bound/free ^3H -leu-enkephalin.



ratio of binding at 0 and 100 mM Na⁺ is about 10 in our experimental conditions.

Correlation between responses of guinea pig ileum and mouse vas deferens

Investigations carried out over several years on a large number of narcotic analgesic drugs have established a good correlation between the relative agonist potencies in the guinea pig ileum and the mouse vas deferens, on the one hand, and their potencies for human analgesia, on the other^{18,23}. Recently, however, parallel assays of certain benzomorphans which are good anti-nociceptive agents but do not substitute for morphine in the morphine-dependent monkey, showed that these compounds can be distinguished by the fact that in the mouse vas deferens their relative agonist potency referred to normorphine as standard is consistently only 25% of that found in guinea pig ileum (Fig. 2)¹⁴. This finding suggests that these compounds, ketocyclazocines and *N*-dimethylfurylbenzomorphans, interact with a receptor which differs from the μ -receptor and is possibly identical with the κ -receptor of Martin and his colleagues^{12,13}. This view is strongly supported by the fact that in both preparations¹⁴ and in the chronic spinal dog²⁴ these compounds require three to six times more naloxone or naltrexone to reverse their agonist action than is needed for the antagonism of morphine and its surrogates which interact with the μ -receptor (compare with Table 2).

These observations on the heterogeneity of the opiate receptors are strongly reinforced by the behaviour of the endogenous opioid peptides. It is a striking feature that LPH₆₁₋₉₁ (C fragment or β -endorphin) has similar ID₅₀ values in the guinea pig ileum and the mouse vas deferens (Fig. 3). On the other hand, no correlation between the two *in vitro* assay models has been found for the shorter peptides. LPH₆₁₋₇₆ (α -endorphin), Met-enkephalin and Leu-enkephalin are progressively more potent in the mouse vas deferens than in the guinea pig ileum; in the latter preparation Met-enkephalin and β -endorphin are about equipotent but α -endorphin and Leu-enkephalin are less active. In contrast to these findings with the peptides, the mouse vas deferens is much less sensitive to the action of morphine than the guinea pig ileum. The possibility has to be considered that these discrepancies between the two *in vitro* preparations may be due to the fact that α - and β -endorphin are resistant to enzymic degradation whereas the enkephalins are rapidly inactivated by peptidases. It seems, however, that this possibility cannot be the main cause because, in the mouse vas deferens, α - and β -endorphin are the least active peptides and, in the guinea pig ileum, α -endorphin is less potent than Met-enkephalin.

From these observations we conclude that the receptor populations in the guinea pig ileum and mouse vas deferens

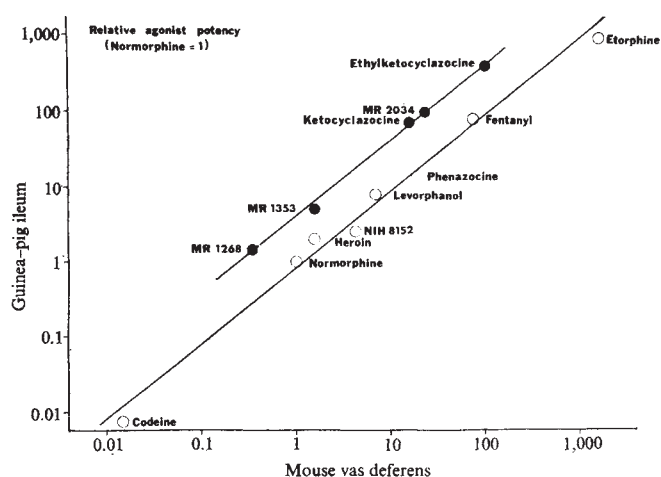


Fig. 2 Relative agonist activities of various narcotic analgesic agonists in the mouse vas deferens and guinea pig ileum (normorphine = 1). The compounds fitting the regression line on the right substitute and those fitting the line on the left do not substitute for morphine in the morphine-dependent monkey. Mr 2034, (—)- α -(1*R*,5*R*,9*R*)-5,9-dimethyl-2-(1-tetrahydrofurfuryl)-2'-hydroxy-6,7-benzomorphane (from Dr H. Merz); Mr 1353, (±)- α -5,9-dimethyl-2-(3-methylfurfuryl)-2'-hydroxy-6,7-benzomorphane; Mr 1268, (±)- α -5,9-dimethyl-2-(2-methyl-3-methylfuryl)-2'-hydroxy-6,7-benzomorphane; NIH 8152, (±)- β -2,9-dimethyl-5-*n*-propyl-2'-hydroxy-6,7-benzomorphane (from Dr E. L. May). (Courtesy of Frances M. Leslie).

are not homogeneous and identical. We now show that the opiate receptors present in homogenates of brain are also heterogeneous.

Inhibition of ³H-leu-enkephalin and ³H-naloxone binding in homogenates of guinea pig brain

When the inhibition of ³H-leu-enkephalin is compared to that of ³H-naloxone binding (Fig. 3), it is found that β -endorphin (LPH₆₁₋₉₁) is equipotent in these two systems. α -endorphin (LPH₆₁₋₇₆) is a weaker inhibitor of ³H-leu-enkephalin binding than β -endorphin; this reduction in potency is even more pronounced for the inhibition of ³H-naloxone binding. Met-enkephalin (LPH₆₁₋₆₅) is about equipotent with β -endorphin against ³H-leu-enkephalin binding but a weaker inhibitor of ³H-naloxone binding in confirmation of earlier observations². Leu-enkephalin is somewhat less potent than β -endorphin as an inhibitor of ³H-leu-enkephalin binding; a particularly interesting finding is its weak inhibition of ³H-naloxone binding against which it has an even lower potency than met-enkephalin. In contrast to the peptides, morphine is a much better inhibitor of

Table 1 Relative inhibitory potencies of Met-enkephalin analogues and β -endorphin on the contractions of guinea pig ileum and mouse vas deferens and on the binding of ³H-naloxone and ³H-leu-enkephalin by brain homogenates

Compound	Guinea pig ileum	Mouse vas deferens	Guinea pig ileum-mouse vas deferens	³ H-naloxone	³ H-leu-enkephalin	Naloxone-Leu-enkephalin
Met-enkephalin	1	1	1	1	1	1
Met-enkephalin amide	1.47 ± 0.18 (3)	0.71 ± 0.08 (3)*	2.1	0.61 ± 0.09 (4)*	0.33 ± 0.02 (3)†	1.8
N-CH ₃ -Met-enkephalin	1.45 ± 0.19 (6)*	0.23 ± 0.01 (5)†	6.3	0.53 ± 0.10 (3)*	0.29 ± 0.03 (4)†	1.8
N-CH ₃ -met-enkephalin amide	3.72 ± 0.96 (5)*	0.33 ± 0.04 (4)†	11	1.20 ± 0.10 (3)	0.08 ± 0.02 (3)†	15
D-Ala ² -met-enkephalin	6.01 ± 0.31 (8)†	5.60 ± 0.62 (7)†	1.1	0.54 ± 0.13 (3)*	1.67 ± 0.45 (3)	0.32

The values are the means ± s.e.; the number of observations are given in parentheses. The relative potencies were determined with Met-enkephalin as internal standard. Met-enkephalin amide and N-CH₃-met-enkephalin amide were provided by Drs A. F. Bradbury and D. G. Smyth. N-CH₃-met-enkephalin and D-Ala²-met-enkephalin were from Dr B. A. Morgan.

**P* < 0.05.

†*P* < 0.001.

^3H -naloxone binding than of ^3H -leu-enkephalin binding. The values obtained for naloxone are not included in Fig. 3 and show the same pattern as morphine, the ID_{50} value against ^3H -leu-enkephalin binding being $51 \pm 11 \text{ nM}$ ($n=5$) and against ^3H -naloxone binding $1.8 \pm 0.5 \text{ nM}$ ($n=3$).

Correlation between receptor affinity in the brain and pharmacological potency in the *in vitro* models

Although β -endorphin is equipotent as an inhibitor of ^3H -leu-enkephalin and ^3H -naloxone binding in guinea pig brain and is also equipotent in the guinea pig ileum and the mouse vas deferens, this relationship does not hold for the other naturally occurring opioid peptides. In guinea pig ileum, the potency pattern of the peptides is similar to that found for the inhibition of ^3H -naloxone binding; morphine, however, is more potent than may be expected from the inhibition of ^3H -naloxone binding. It has already been pointed out that, in the mouse vas deferens, α -endorphin, met-enkephalin and Leu-enkephalin are in this order progressively more active than β -endorphin, a pattern fundamentally different from that of inhibition of ^3H -naloxone binding; there is less discrepancy between the order of potency and the values found for the inhibition of ^3H -leu-enkephalin binding. Morphine is a weak inhibitor of ^3H -leu-enkephalin binding which agrees with its weak agonist activity in the mouse vas deferens. It seems, therefore, that in principle, the activity in the guinea pig ileum is better correlated to inhibition of ^3H -naloxone binding than to inhibition of ^3H -leu-enkephalin binding. In contrast, activity in the mouse vas deferens is not correlated to inhibition of ^3H -naloxone binding but seems to have a certain similarity to the pattern of the inhibition of ^3H -leu-enkephalin binding.

This relationship between the binding assays and the *in vitro* preparations is further illustrated when the binding and pharmacological properties of Met-enkephalin are changed by minor modifications of the molecule (Table 1). In the guinea pig ileum, amidation of the carboxyl group of the C-terminal methionine or the introduction of a methyl group in the amino group of the N-terminal tyrosine increases potency a little whereas in the mouse vas deferens activity is decreased, particularly of the latter analogue. The inhibition of ^3H -naloxone binding is about halved by both analogues and that of ^3H -leu-enkephalin reduced by about 70%. A combination of the two modifications yields $N\text{-CH}_3\text{-Tyr-Gly-Gly-Phe-Met-NH}_2$, with characteristics very different from its parent compound: it is almost four times more potent in the guinea pig ileum and only one-third as potent in the mouse vas deferens; it inhibits ^3H -naloxone binding as well as Met-enkephalin but has lost over 90% of its ability to inhibit ^3H -leu-enkephalin binding. Since the potency of Met-enkephalin is about eight times greater in

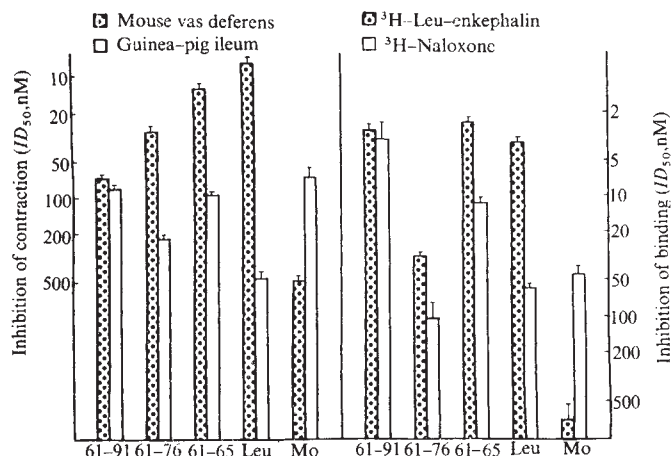


Fig. 3 The agonist activities of various compounds in the mouse vas deferens and guinea pig ileum and their potencies to inhibit ^3H -leu-enkephalin and ^3H -naloxone binding in homogenates of guinea pig brain ($\text{pH } 7.4$ at 0°C , no Na^+ , 150 min). The numbers on the abscissa indicate the amino acid sequence of β -lipotropin; Leu, leu-enkephalin and Mo, morphine. All peptides are synthetic (61-91, ovine β -endorphin, Dr C. H. Li; 61-76, porcine α -endorphin, Dr R. Guillemin; 61-65, met-enkephalin and leu-enkephalin, Dr B. A. Morgan).

the mouse vas deferens than in the guinea pig ileum, $N\text{-CH}_3\text{-met-enkephalin amide}$ is about 50% more potent in the guinea pig ileum than in the mouse vas deferens; further, since met-enkephalin inhibits ^3H -leu-enkephalin binding about five times more effectively than ^3H -naloxone binding, $N\text{-CH}_3\text{-met-enkephalin amide}$ is three times more potent against ^3H -naloxone than against ^3H -leu-enkephalin. A different picture is obtained when in met-enkephalin glycine² is replaced by the unnatural amino acid D-alanine. Compared with its parent compound, this analogue shows a sixfold increase in potency in both preparations, and is therefore about eight times more potent in the mouse vas deferens than in the guinea pig ileum. Its ability to inhibit ^3H -naloxone binding is halved but there is no reduction in its potency against ^3H -leu-enkephalin which therefore is inhibited about 15 times more than ^3H -naloxone. Thus, $\text{D-Ala}^2\text{-met-enkephalin}$ has a pattern of activity similar to that found for its parent compound, whereas $N\text{-CH}_3\text{-met-enkephalin amide}$ has in its relative, but not in its absolute, potencies acquired a pattern reminiscent of that of morphine. Both compounds show considerable resistance to inactivation by peptidases and after intraventricular or intracerebral administration exhibit antinociceptive activity^{25,26} which therefore does not seem to be closely and exclusively related to their affinity to receptors represented by either naloxone or leucine-enkephalin binding. $\text{D-Ala}^2\text{-met-enkephalin}$ is about equipotent with β -endorphin as an

Table 2 The effectiveness (K_e , nM) of naloxone and Mr 2266 to antagonise the agonist actions of normorphine, enkephalins and benzomorphans without physical dependence capacity

Compound	Naloxone*		Mr 2266†	
	Guinea pig ileum	Mouse vas deferens	Guinea pig ileum	Mouse vas deferens
Normorphine	2.64 ± 0.04 (3)	1.84 ± 0.20 (4)	1.93 ± 0.24 (4)	1.50 ± 0.14 (5)
Met-enkephalin	2.45 ± 0.26 (4)	22.6 ± 0.46 (4)	3.58 ± 0.08 (3)	16.2 ± 1.5 (4)
Leu-enkephalin	2.43 ± 0.67 (4)	21.4 ± 3.3 (4)	2.36 ± 0.44 (3)	34.0 ± 5.3 (6)
Normorphine	1.89 ± 0.15 (12)	3.10 ± 0.25 (6)	1.93 ± 0.24 (4)	1.50 ± 0.14 (5)
($\pm$)-Ethylketocyclazocine	14.9 ± 0.9 (4)	11.0 ± 0.6 (3)	2.53 ± 0.60 (4)	4.51 ± 0.54 (6)
Mr 2034	10.4 ± 1.8 (7)	9.1 ± 0.7 (3)	3.34 ± 0.44 (3)	8.4 ± 1.3 (3)

Values are the means $\pm$ s.e.; the number of observations are given in parentheses.

*The values were obtained by a modification¹⁶ of the method of Arunlakshana and Schild²⁸ on segments of ileum. In the myenteric plexus-longitudinal muscle preparation K_e values were obtained by the 'single dose' method¹⁶: normorphine, $1.89 \pm 0.20 \text{ nM}$ (4), ($\pm$)-ethylketocyclazocine (Dr S. Archer) $11.9 \pm 1.2 \text{ nM}$ (3) and Mr 2034 $11.9 \pm 1.7 \text{ nM}$ (4).

†Mr 2266 (Dr H. Merz) has some agonist activity so the 'single-dose' method was used on the myenteric plexus-longitudinal muscle preparation and the mouse vas deferens.

antinociceptive agent in the rat²⁷ whereas *N*-CH₃-Met-enkephalin amide is less potent in the cat²⁸.

Differentiation of opioid receptors by action of antagonists

As explained previously, receptors of different binding properties present in a mixed receptor population are best differentiated by antagonists which are specific for the different receptors. The effectiveness of an antagonist against a given agonist is measured by the equilibrium dissociation constant K_e ; the value of this constant is equal to the antagonist concentration which requires a doubling of the agonist concentration to give the same pharmacological response as that obtained in the absence of any antagonist. K_e may also be expressed as its negative logarithm, pA_2 .

In the guinea pig ileum, the enkephalins are as readily antagonised by naloxone as is normorphine (Table 2). In mouse vas deferens, however, about 10 times more naloxone is required for the antagonism of the enkephalins than for that of normorphine. This low antagonist potency of naloxone in the mouse vas deferens seems to hold for all opioid peptides (Table 3), irrespective of whether they are more active in mouse vas deferens than in guinea pig ileum (for example, the enkephalins, α -endorphin and D-Ala²-Met-enkephalin), or not (β -endorphin and *N*-CH₃-Met-enkephalin amide).

These observations strongly reinforce the conclusions made from the pharmacological responses to the enkephalins and the endorphins, namely that their action in the mouse vas deferens is on receptors different from those on which morphine and its classical surrogates act. It is important to note that these receptors, which may be called δ -receptors⁸, are not responsible for the action of the enkephalins in guinea pig ileum. Since in this preparation the enkephalins are antagonised by naloxone as readily as morphine, they may be assumed to act on morphine- or μ -receptors.

As far as specific antagonists are concerned, no such antagonist has been found for the putative δ -receptor although the oripavine derivative diprenorphine is relatively more effective than naloxone⁹. The search for an antagonist acting on the putative κ -receptor has been somewhat more successful. In the guinea-pig ileum Mr 2266 is a potent antagonist of compounds acting on μ -receptors (for example, normorphine and the enkephalins) and also of the putative κ -agonists, ethylketocyclazocine and Mr 2034 (Table 2, see Fig. 2 legend for Mr 2034 structure). It seems from these experiments in the guinea pig ileum that, in contrast to naloxone, Mr 2266 acts equally well on μ - and κ -receptors. In the mouse vas deferens, however, Mr 2266

is not more effective as an κ -antagonist than naloxone. This difference between the two preparations is difficult to explain unless one assumes that there are only few κ -receptors in the mouse vas deferens whose relative insensitivity to putative κ -agonists has already been discussed.

Summary of evidence of opioid receptors

Investigation of the pharmacological responses of the guinea pig ileum and the mouse vas deferens to the inhibitory effects of the opioid peptides have led to the view that the receptor populations in the two *in vitro* models are heterogeneous and not identical. In the guinea pig ileum, the peptides interact mainly with the μ -receptor which mediates the action of the classical morphine-like compounds. In the mouse vas deferens, on the other hand, the opioid peptides interact mainly with the putative δ -receptors although they probably also affect the μ -receptors. It would seem likely that the δ -receptors are related to the binding sites in the brain for which ³H-leu-enkephalin has a high affinity while the μ -receptors may be related to the binding sites in the brain and the myenteric plexus of the guinea pig ileum which have a high affinity to ³H-naloxone^{19,29} and to ³H-dihydromorphine^{30,31}.

The guinea pig ileum also has receptors which are probably identical with the κ -receptors first postulated from the results of experiments on the chronic spinal dog^{12,13}; the mouse vas deferens seems to have fewer κ -receptors than the guinea pig ileum. It is not certain whether or not κ -receptors have a role in the action of the endogenous opioid peptides.

In view of the complexity of the opioid receptors, the use of a single assay system can no longer be considered a reliable method for prediction of the pharmacological activity of new opioid compounds, particularly of those with a peptide structure. For such compounds, multiple parallel assays similar to those used in the present investigation may be useful.

Implications of multiple receptors for physiological functions of opioid peptides

One of the most interesting findings has been the fact that β -endorphin inhibits equally well ³H-leu-enkephalin and ³H-naloxone binding, and is also equipotent in the guinea-pig ileum and mouse vas deferens. This observation may be interpreted as indicating that β -endorphin, which is possibly a precursor of other opioid peptides, interacts equally well with several or perhaps all opiate receptors. The shorter peptide, Met-enkephalin, is in several aspects different from its putative parent substance in that it has greater affinity to the binding site of ³H-leu-enkephalin than to that of ³H-naloxone, that it is more potent in the mouse vas deferens than in the guinea pig ileum and, finally, that it is readily inactivated by peptidases. Thus, it seems that Met-enkephalin may be designed for a more specialised function than β -endorphin.

The evidence available at present is insufficient to attempt to allocate different receptors to different physiological functions. We have already pointed out that antinociceptive action does not seem to be exclusively associated with any one receptor. It is, however, apparent that the opioid peptidergic system is of considerable complexity in that several agonists are matched by multiple receptors. These circumstances are similar to those found in the catecholamine system, but whereas in the latter system transmitter action is terminated by neuronal re-uptake, the opioid peptides are rapidly inactivated by enzymatic degradation, as is acetylcholine in the cholinergic system. Is it permissible to compare the neuroendocrine element of the catecholamine system, adrenaline, with the member of the peptidergic system, β -endorphin, which may also have a neuroendocrine function? Adrenaline is present in high

Table 3 Effectiveness of naloxone in antagonising the agonist actions of normorphine and opioid peptides in guinea pig ileum and mouse vas deferens

Agonist	Guinea pig ileum K_e (nM)	Mouse vas deferens K_e (nM)
Normorphine	1.89 ± 0.20 (4)	1.84 ± 0.20 (4)
Tyr-Gly-Gly-Phe	1.73, 1.77	29.2 ± 2.7 (4)
Met-enkephalin	2.45 ± 0.26 (4)	22.6 ± 0.5 (4)
α -Endorphin (61-76)	1.56 ± 0.15 (4)	22.5 ± 2.9 (4)
β -Endorphin (61-91)	2.86 ± 0.42 (5)	21.7 ± 1.2 (3)
<i>N</i> -CH ₃ -Met-enkephalin	2.07 ± 0.08 (4)	15.6 ± 3.8 (4)
<i>N</i> -CH ₃ -Met-enkephalin amide		16.6 ± 2.6 (4)
D-Ala ² -Met-enkephalin	2.55 ± 0.20 (4)	22.4 ± 2.7 (4)

K_e values were determined by a modification¹⁶ of the method of Arunlakshana and Schild²⁸ or the 'single dose' method¹⁶ and are the means ± s.e.; the number of observations are given in parentheses. Tyr-Gly-Gly-Phe was provided by Dr B. A. Morgan.

concentration in the adrenal medulla and β -endorphin and α -endorphin are found in the intermediate and anterior lobes of the pituitary gland, also in high concentrations³². Adrenaline is essential for the fight and flight reaction. β -Endorphin may be of similar importance: could the often reported lack of pain perception on the battlefield or the reduced pain experienced during childbirth be explained on this basis? In contrast, the other components of the two systems, noradrenaline and dopamine on the one hand, and Met-enkephalin and Leu-enkephalin on the other, are present in lower concentrations in widely-spread areas of the central and autonomic nervous systems^{33,34}. Immunohistochemical studies show that Met-enkephalin and Leu-enkephalin are widely distributed in neuronal fibres and terminals of the central nervous system of the rat^{35,36}. These findings and the rapid inactivation of the enkephalins are compatible with the role of neuromodulator or neurotransmitter suggested for them^{34,37}. If this concept is correct, it would follow that the enkephalins would subserve transmission of a rapid and more or less transient character while the longer-chain peptides, particularly β -endorphin would mediate neuronal and possible endocrine changes of a more lasting nature.

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letters to nature

Comments on the optical and radio detection of black hole explosions

It has recently been suggested¹ that it may be easier to detect the explosions of miniholes by the radio and optical pulses produced by the interaction of the emitted particles with the interstellar magnetic fields, than by the direct detection of the high energy γ rays².

We concern ourselves here first with the possibilities in the optical band. In the several attempts in recent years to detect fast transient phenomena in the visible band^{3–5}, including searches for X-ray bursts by the upper air atmospheric fluorescence technique^{6,7} the time constants have covered the region $\sim 100 \mu\text{s}$ to 10 s. Most of these experiments have been bedevilled by interference of one form or another, both man-made and natural.

The radiation mechanism proposed by Rees¹ is unique in that its timescale is far shorter than that for any other known phenomenon in the optical band. The pulse width or at least the rise time, for emission at a characteristic wavelength λ , on one of the models discussed, will be $\sim (\lambda/c)$ or $\sim 10^{-15}$ s. The shock front will be broadened by dispersion, both in the interstellar plasma and in the terrestrial atmosphere. The smearing due to the former, normally considered insignificant in the optical region, amounts to $\sim 5 \times 10^{-13}$ s over the band $\lambda = 400$ –600 nm, if we accept a typical but arbitrarily chosen value of $DM = \int n_e d \sim 50$ electrons cm^{-3} pc. The atmospheric dispersion is $\sim 2 \times 10^{-10}$ s for a scale height of 7 km and a

variance of 2.5% in $(n-1)$ over the same bandwidth. In either case, however, the timescales are much shorter than the response times of existing detectors, ~ 1 ns for small phototubes and ~ 10 ns for larger ones.

An optical search for shocks from minihole explosions has two practical advantages over radio. First, the fractional bandwidth which can be used is large, ~ 0.4 , and second, in the optical band, the only known sources of pulses on these timescales arise from Cerenkov flashes produced by extensive cosmic-ray showers⁸. This contrasts markedly with the situation at radio frequencies in which bandwidth-limited interference pulses are always observed, though in this case dispersion can be used to advantage for a celestial source.

If S_0 is the source strength in the optical band and we demand that the detected signal, on a timescale τ , shall exceed the threshold sensitivity set by the night-sky background noise, the distance d at which a single black hole explosion would be detected by a phototube of given quantum efficiency, with a mirror of effective area A and acceptance cone Ω , is

$$d = (S_0/4\pi)^{0.5} \text{const} (A^{0.25} \Omega^{-0.25}) \quad (1)$$

Since, however, the locations of the explosions are unknown, we must assume they are uniformly distributed and occur randomly over time. In this case, the observable volume will be

$$V = (\Omega d^3/3) = \text{const}' (A^{0.75} \Omega^{+0.25}) \quad (2)$$

From this we conclude that we require a flux collector

rather than a telescope, one with the largest collecting area and acceptance cone. The installations which have been used so extensively in high energy γ -ray astronomy⁹ therefore fulfil the requirements. In this technique, based on the Cerenkov effect of extensive cosmic-ray showers in the atmosphere⁸ resolving times τ of ~ 10 ns are in regular use and A and Ω are both large. For the large 10-m optical reflector at Mount Hopkins¹⁰, $A = 60 \text{ m}^2$ and $\Omega = 1.5 \times 10^{-4} \text{ sr}$ (ref. 7, p336). It is this flux collector which has already been used¹¹ to search for the γ -ray bursts originally predicted from minihole explosions².

The first example suggested in ref. 1, in which 10^{18} J might be emitted optically, in the case for $\gamma \sim 300$, gives an unfavourable prediction since the radiation from the fireball will be incoherent, and the duration of the pulse $\sim 0.1 \text{ s}$, just the time domain in which the interference problems are serious. It has, however, more recently been pointed out to us (M. J. Rees, personal communication) that the particle physics may be such that $E_{\text{opt}} \sim 10^{23} \text{ J}$, $\gamma \sim 10^7$ and hence that the radiation would then be coherent. In this case the prospects look much better. Taking the sensitivity of the 10-m reflector to be 5 photon m^{-2} (ref. 7, p.336) an explosion of 10^{23} J at $\lambda \sim 450 \text{ nm}$, for $\tau \sim 10 \text{ ns}$, would be detectable out to $d \sim 2 \text{ kpc}$ for which, from equation (2), $V \sim 4 \times 10^5 (\text{pc})^3$.

A multiplicity of spaced flux collectors would, however, be required, to suppress the chance-rate arising from Cerenkov flashes¹¹ from extensive cosmic-ray showers and other local phenomena. The intrinsic threshold detection rate for pbh explosions of this type would be $\sim 4.10^{-5} \text{ yr}^{-1} (\text{pc})^{-3}$ for the above volume and an observing time of $\sim 500 \text{ h}$. To achieve such a limit however, T. C. Weekes (personal communication) has pointed out that about five stations would be required, for an air-shower rate $\sim 10 \text{ s}^{-1}$ at each station and a coincidence resolving time $\sim 1 \text{ ms}$.

Turning now to the question of detecting radio bursts from pbh explosions, the situation is different, in that certain limits can already be set from experiments in other areas. In the radio region, the relationship between A and Ω is fixed, by the coherence properties of antennas.

If the energy emitted by a minihole explosion in unit bandwidth in the radio region is S_R , then the maximum distance for the detection of such an explosion is given by $d_{\text{max}} = (S_R/4\pi\epsilon_R)^{0.5}$ where ϵ_R is the energy threshold per unit area per unit bandwidth of an actual receiver system. For a single radio receiving system working at a specified frequency, $A\Omega$ is a constant, and $\epsilon_R \propto A^{-1}$, so that in the radio case, the corresponding expression to (2) is

$$V = \text{const}'' A^{0.5} \quad (3)$$

The achievable limits on the number of miniholes producing radio pulses is then proportional to $(A^{0.5}T)^{-1}$ where T is the total observing time of the experiment. The many radio experiments quoted in the literature can be divided broadly in two classes; firstly those involving small wide field of view antennas at low frequencies run for long periods of time, and secondly, those involving larger directional antennas at higher frequencies usually run for shorter periods. The duration of the emitted pulse is not a dominant consideration in most of the experiments as, provided the pulse duration, to include broadening by dispersion, is less than the system integration time, it is only the total energy in the pulse that determines the threshold level.

The characteristic frequency ν_c for the radio emission estimated by Rees, varies as a high power of γ_t ($\sim 2 \times 10^{16} \text{ g } M_{\text{crit}}^{-1}$); for $\gamma = 10^5$, $\nu_c \sim 3 \text{ GHz}$. Without knowing the values of γ , the optimum frequency cannot be selected in advance. We have calculated therefore the upper limits on the rates of such explosions, R , (99.9% confidence level) for two representative systems; one in each of the above classes (Table 1). In comparing these limits with those obtained in the γ -ray searches¹¹ it must be borne in mind that the radio and optical emission predicted by Rees is critically sensitive to

Table 1 Upper limits on the rates of explosions for two representative systems

System	ν (MHz)	$\delta\nu$ (MHz)	Antenna	Ω (sr)	Duration of experiment (h)	R ($\text{pc}^{-3}\text{yr}^{-1}$)
Small wide field of view antennas run for long periods ¹²	151	1	Dipoles	~ 1.3	3620	7×10^{-4}
Larger directional antenna run at a higher frequency for shorter periods ¹³	1665	15	25.6m dish	2.2×10^{-4}	28	0.1

the value of γ_t , which depends on the particle physics assumed.

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Optical pulses from primordial black hole explosions

REES¹ has pointed out recently that explosions of small black holes² could be detected by the radio or optical emission from an expanding relativistic shock wave interacting with the ambient magnetic field. The frequency of the radiation is determined by the value of a parameter $\gamma_t = (2 \times 10^{16} \text{ g}/M_{\text{crit}})$, where M_{crit} is the mass at which the black hole explodes. Jelley *et al.*³ have considered the optical emission and the radio emission which will occur for $\gamma_t \sim 10^5$, and shown that upper limits can be set for the rate of explosions, from previous measurements at 150 and 1,700 MHz.

For a lower value of γ_t , corresponding to the nuclear model of Hagedorn⁴, optical emission will be produced, but it will be incoherent, and the fraction of the total energy appearing is small. A limit has been set for γ rays for this model, using the atmospheric Cerenkov technique, by Porter and Weekes⁵. As Jelley *et al.* have pointed out, Cerenkov systems are suitable also for detecting fast optical pulses. For a γ_t value of 10^7 , 10^{30} erg of optical radiation

are predicted by ref. 1. This would correspond to a nuclear model at the opposite extreme from the Hagedorn model, and it is not clear whether the black hole explosion could occur in a sufficiently short time to give the required shock front.

If such an optical pulse is produced, however, the measurements made in ref. 5 can be used to provide an upper limit for the explosion rate. The optical sensitivity of the detectors was 370 photons m^{-2} corresponding to 1.6×10^{-13} erg cm^{-2} . The maximum distance for detection is then $r = E^{1/2}/2\pi^{1/2}F^{1/2}$, where E is the total energy produced and F the optical sensitivity. With $E = 10^{30}$ erg, r is 7×10^{20} cm, or 230 pc. With the solid angle of 4×10^{-3} sr available, this gives a sensitive volume of 1.5×10^4 pc³.

The observed one event in 15.3 h of observation⁵, which is compatible with random expectation, corresponds to an upper limit of 6.8 events at the 99% confidence level. This yields an upper limit of 3.9×10^3 events yr^{-1} and hence an upper limit for the explosion rate of 0.3 events $\text{pc}^{-3} \text{yr}^{-1}$. The limit found for γ rays was of the same order, but this was, of course, for the Hagedorn or Composite Particle model, in which 10^{33} erg are released at the explosion.

The sensitivity of this experiment could be improved by the use of a larger array of separated detectors, to reduce the chance rate, and a longer running time. In order to reach the sensitivity quoted by Jelley *et al.*, however, at least four very large optical reflectors each of aperture greater than 10 m and separated by more than 10 km, would be required. This calculation assumes a coincidence resolving time of 10^{-4} s and a running time of 500 h.

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Neutrino angular momentum losses in stellar collapse

We know from observation that even the most rapidly rotating neutron stars—those of the Crab and Vela—are slow rotators, in the sense that their angular momentum is of negligible structural importance. In fact, at least for these pulsars, estimates based on their current slowdown rates indicate their rotation frequencies at birth were probably only a few times higher than the present ones¹, even though the main sequence progenitors probably had much more angular momentum per unit mass. The purpose of the present note is to examine the degree to which one can attribute such a decrease in the angular momentum per unit mass to neutrino radiation emitted during the formation of the neutron star from the collapse of a sufficiently massive stellar core.

According to the current theory, pulsars are presumably neutron stars which originate from the collapse of the cores of sufficiently massive stars ($M \geq 7M_{\odot}$) (ref. 2). After thermo-

nuclear burning has stopped, such stellar cores reach a state of very high density and temperature, so that the electron chemical potential is high enough to favour electron absorption on to protons ($e^-p \rightarrow n\nu_e$) over the reverse reaction (β decay). Electrons are thus eliminated, and so their contribution to the pressure decreases, causing the core to collapse. At the same time, vast amounts of neutrinos are produced by the inverse β decay and other thermal processes, taking up all the gain in gravitational energy arising from the collapse. The core finally settles down at densities of the order of 10^{14} – 10^{15} g cm^{-3} , it is then supported by nuclear degeneracy. One expects the stellar core to be rotating, increasing its angular velocity while collapsing by conserving angular momentum. The neutrinos leaving the core are emitted from a surface shell, approximately one mean free path thick. These neutrinos were initially produced throughout the core and not necessarily near its surface. If the rotation of the core is uniform, neutrinos of low angular momentum regions interact before escaping with the high angular momentum material of the surface. The result of such an interaction will be the neutrinos will gain angular momentum and so the angular momentum per unit mass of the escaping core will fall.

Let us consider a simple model, which allows analytic calculations and includes the general features of the actual situation, in order to estimate the effect neutrinos can have on the rotation of such a collapsing core. Consider a homogeneous spherically symmetric and uniformly rotating core, collapsing from density $\sim 10^{12}$ g cm^{-3} to 10^{15} g cm^{-3} , and neutrinos take away all the gain in potential energy arising from the collapse.

In this core consider a spherical shell between r and $r+l$ (Fig. 1) ($r \gg l$, $l \approx$ neutrino mean free path). Let $\dot{E}(r)$ be the

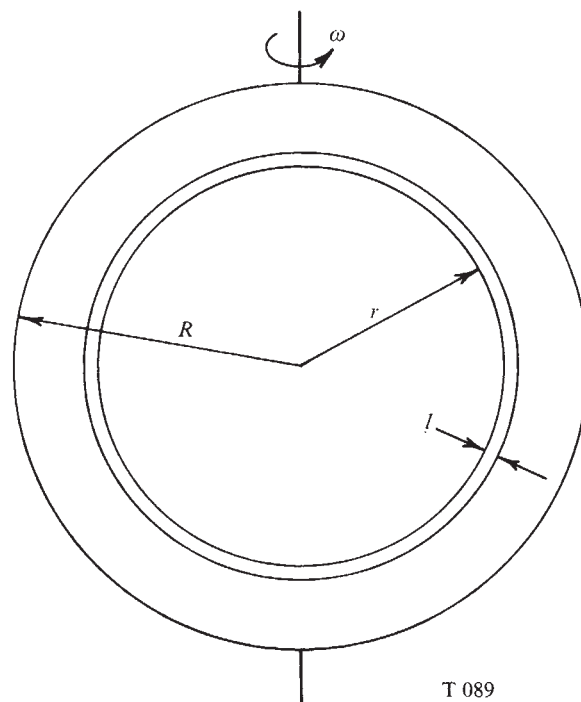


Fig. 1 Section through a burnt-out stellar core.

rate at which energy in the form of neutrinos is leaving the mass inside radius r . These neutrinos are emitted isotropically in the fluid's rest frame. Owing to the relativistic 'head light effect'³ from an inertial frame emission will seem to take place preferentially along the direction of rotation, resulting in a net angular momentum loss. This loss is further enhanced by a Doppler shift in the energy of the forward-against the backward-

emitted neutrinos. It is then easy to show that the part of the core inside radius r is losing angular momentum at a rate

$$\dot{J}(r) = -\frac{7}{9} \frac{\dot{E}(r)}{c^2} \omega r^2 \quad (1)$$

where ω is the angular velocity of rotation. These neutrinos and the corresponding angular momentum, are of course deposited in the shell between r and $r+l$, and then re-emitted at radius $r+l$, raking away angular momentum at a rate $J(r+l)$. The net effect on the shell, therefore, will be to lose angular momentum at a rate

$$\dot{J}(r) - \dot{J}(r+l) = -\frac{7}{9} \frac{\dot{E}(r)}{c^2} 2\omega r l + O\left(\frac{l}{r}\right)^2 \quad (2)$$

Summing up the angular momentum loss rate over all shells, the total angular momentum loss rate is found to be

$$\dot{J}(R) = -\frac{1}{3} \frac{7}{9} \frac{\dot{E}(R)}{c^2} \omega R^2 \quad (3)$$

where R is the core radius and $\dot{E}(R)$ the total energy loss rate, which for the homogeneous sphere assumed in our case is

$$\dot{E}(R) = -\frac{3GM^2}{5R^2} \dot{R}$$

where M is the mass of the core.

The evolution now of angular velocity ω with R as the core collapses is easy to be followed by the equation

$$I\dot{\omega} + I\dot{\omega} = \dot{J} \quad (4)$$

where $I = 2/5 MR^2$, is the moment of inertia of the collapsing core. Straightforward integration of equation (4) gives

$$\omega = \omega_0 \left(\frac{R_0}{R}\right)^2 \exp\left[-\frac{1}{2} \frac{7}{9} \frac{GM}{c^2 R_0} \left(\frac{R_0}{R} - 1\right)\right] \quad (5)$$

(The subscript zero denotes the initial values of the corresponding quantities.)

The loss of neutrino angular momentum affects only the value of the exponential factor in equation (5) (see Fig. 2). Since the mass of the collapsing core and the ratio of its initial to final radius determine the total energy available to neutrinos for slowdown, it is not surprising that the neutrino angular momentum depends on them.

If we substitute realistic values for these quantities we can estimate how big the effect should be. For $R_0/R = 10$ and $M = 1.5M_\odot$ we find the value of the exponential factor to be 0.91. If we take $M = 2.5M_\odot$, which is close to the highest non-rotating neutron star mass allowed by an asymptotic free quark equation of state (Kislinger & Morley, personal communication), then its value is reduced to 0.88. So neutrinos seem to decrease ω from its expected value $\omega_0 (R_0/R)^2$ by 9% and 12% respectively for the two masses considered here. At even higher masses cores collapse to black holes. The magnitude of the effect then reaches a maximum ($\sim 18\%$ decrease), independent of the mass of the collapsing core, corresponding to $R = r_s = 2GM/c^2$ (the Schwarzschild radius) and $R_0 \rightarrow \infty$.

We have shown that neutrinos tend to slow down the rotation of a collapsing stellar core by carrying away part of its angular momentum. The effect seems to be present in all rotating collapsing cores, but does not completely account for the slow rotation of the compact supernova remnants (pulsars). But the angular momentum taken away by neutrinos and deposited in

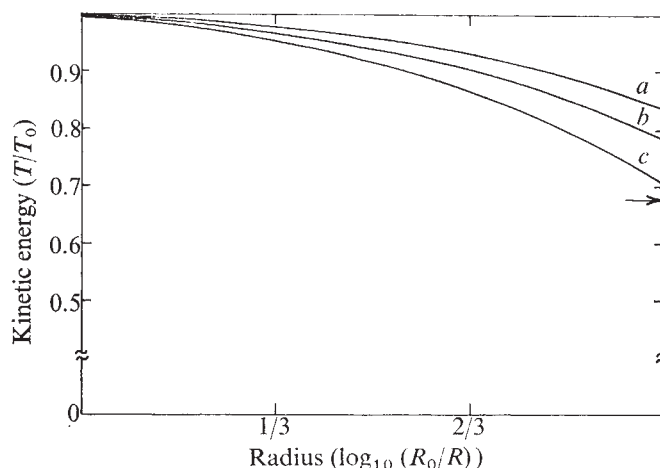


Fig. 2 The kinetic energy of the collapsing core T , measured at any instant in units of its value if no angular momentum losses are present T_0 , as a function of the core radius. *a*, For $M = 1.5M_\odot$ and $R/R_0 = 0.1$; *b* for $M = 2.5M_\odot$ and $R/R_0 = 0.10$; *c*, collapse to a black hole with $r_s/R_0 = 0.1$. The arrow indicates the (minimum) value of the ratio T/T_0 in the case that $r_s/R_0 \rightarrow 0$.

the expanding envelope may have some observational effects (that is, axial rather than spherical symmetry of the ejecta) or may help the explosion. Generalisation also of the above spherically symmetric treatment to sufficiently slowly rotating McLaurin spheroids gives essentially the same results. If we do not assume that rotation is uniform, the slowdown effect is smaller. In such a case neutrinos will act as sort of radiative viscosity smoothing out inequalities in the angular velocity.

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Small scale auroral absorption events associated with substorms

We describe the first results obtained from a new facility designed to study auroral absorption events exhibiting small scale spatial structure and rapid temporal variations. The absorption is measured by means of a modern solid-state riometer with high time resolution¹ and the spatial resolution is obtained using a narrow-beam antenna with 64 elements. The most striking observations made so far involve intense, short-lived 'spikes' of absorption associated with the onsets of auroral substorms. These spikes, which seem to be of small spatial scale, are probably related to the sudden ionospheric absorption (SIA or SAI) events reported by previous workers²⁻⁵ and perhaps to the 'spikes' of intense energetic electron precipitation observed from polar-orbiting satellites⁶⁻¹¹. Five such spikes were observed during November and December 1975.

The antenna array is constructed of elements made of coaxial cable (RG-62) with the inner and outer conductors interchanged at half-wavelength intervals¹². Eight such elements, each equivalent to eight half-wave dipoles, are mounted with a half wavelength between adjacent elements and a quarter wavelength above a ground plane. The antenna was first set up in Lindau and its radiation pattern determined by flying a portable transmitter through it. The predicted and observed patterns are in reasonably good agreement with $\sim 16^\circ$ between half-power points and with the side-lobes suppressed about 10 dB below the main beam (see Fig. 1). A similar array was erected in Ramfjord ($L \sim 6.2$) near Tromsø in northern Norway at the end of 1975. The riometer, which operates at a frequency of 51.4 MHz, has a band width of 150 kHz and a time constant of 0.25 s for both increasing and decreasing signal intensities¹.

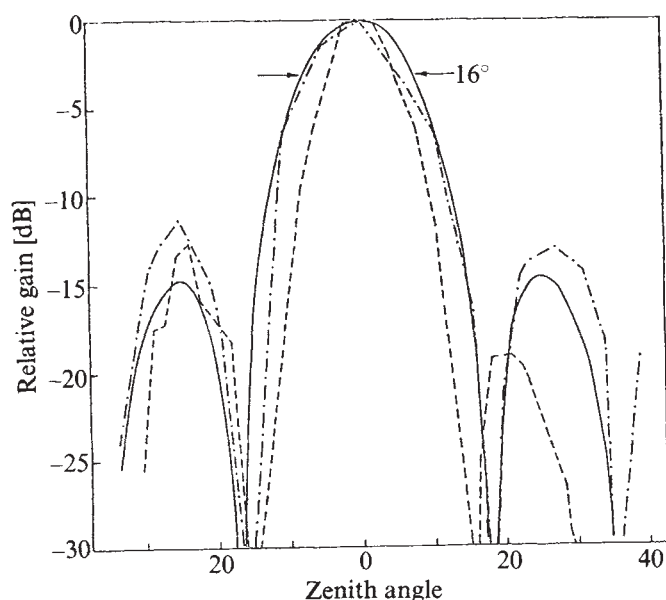


Fig. 1 Power polar diagram of the riometer antenna. The full-drawn curve represents the calculated pattern, while the two other curves are results of measurements of the directional variation of the antenna gain. The transmitting antenna used in these measurements was a crossed Herizian dipole taped on to the underside of the non-metallic body of an aircraft.

An example of one of the more impressive spikes observed during the first few months of operation of the system is shown in Fig. 2a. The absorption is shown as the equivalent absorption at 30 MHz assuming that the absorption varies inversely as the square of the frequency. The absorption observed simultaneously from a broad-beam 30-MHz riometer is also shown for comparison. The spike has a duration of approximately 1 min and the maximum absorption at 51.4 MHz is ~ 8 dB, corresponding to ~ 25 dB at 30 MHz. The maximum absorption measured by the broad-beam 30-MHz system was significantly smaller (~ 7.5 dB), which suggests that at the time the absorbing region did not completely fill the beam. Following the spike, there is a good correspondence between the two sets of observations. This indicates that the region of electron density enhancement in the D layer which produced the absorption had a spatial scale comparable with, or larger than, the field of view of the antennas and that the bulk of the absorption occurs at an altitude where the signal frequency is much larger than the electron-neutral collision frequency (that is, $\gtrsim 60$ km).

The absorption spike, which occurred at ~ 1750 UT on 3 November 1975, is coincident with the very sharp onset of a negative bay ($\sim 680\gamma$, Fig. 2b) and a rapid declination variation (~ 150 arc s), both of which had time scales

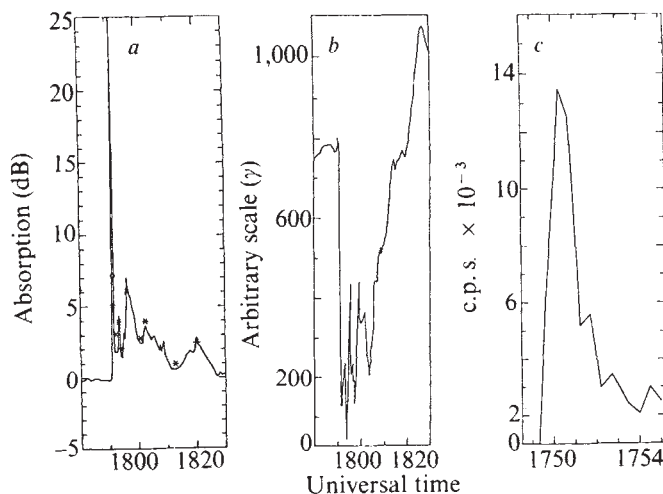


Fig. 2 *a*, An example of a very intense spike in ionospheric absorption occurring at Ramfjord at ~ 1750 UT on 3 November 1975. The circle and the stars indicate absorption measured using a broad-beam 30-MHz antenna (P. Stauning, personal communication). The absorption measured directly is marked with a circle, while absorption measurements corrected for zenith angle effects are marked with a star. This correction is made with the assumption that the sky temperature and absorption are homogeneous. *b*, Storm magnetogram from Tromsø showing H component of magnetic field (A. Brekke, personal communication). The absorption event occurred during the negative bay. *c*, ATS-6 36-keV electron measurements (C. E. McIlwain, personal communication). The counting rate started to increase about 1 min before the absorption, which may indicate that the magnetic conjugate point to ATS-6 was located south of Ramfjord before the absorption event.

of the order of 1 min. The substorm onset was observed simultaneously by energetic particle detectors on the geostationary Earth satellite ATS-6, which at the time has its geomagnetic conjugate point located 0.9° east and 1.0° south of our ground station in Ramfjord¹³. As observed at the satellite, the substorm was characterised by an increase by a factor of ~ 200 in the intensity of 36 keV electrons which took place with a timescale of the order of 1 min at 1750 UT. The electron intensity was observed to decay by a factor of about 10 during the next few minutes, so that in effect an electron spike was observed with a time duration of the order of 1 min. The 36-keV electron count rate is shown on a linear scale in Fig. 2c for comparison with the absorption measurements.

It is not completely clear how these observations can be interpreted in the absence of other evidence. One possibility, however, is that an absorbing region, spatially restricted in one direction but extended in the other, passed rapidly across the point of observation. If we assume that the vertical pencil beam absorption has the form $A(x) = A_0 \exp(-x^2/2x_0^2)$, where x is the horizontal distance from the line of maximum absorption, then, using both narrow- and broad-beam absorption measurements, one finds that the width of the absorbing region ($2x_0$) must have been about 50 km or 0.5 degrees of latitude if the absorption is assumed to occur at an altitude of ~ 80 km. Simultaneous broad-beam observations at 30 MHz and 40 MHz are consistent with this interpretation (P. Stauning, personal communication). The spatial scale we have deduced is consistent with the latitudinal width of energetic particle spikes observed by low-altitude satellites⁸⁻¹¹. Since the absorbing region must pass through the riometer antenna beam and intersect the conjugate point to ATS-6 within a time span of ~ 1 min at the most, the horizontal speed of the absorbing region must be quite considerable (that is, ~ 1 –10 km

s^{-1}). This speed is similar to that of the poleward surge of auroral substorms¹⁴. Unfortunately we are unable to determine from the available all-sky camera records (one picture every 5 min) whether or not an arc-like structure passed rapidly over our observation point at the time in question.

An alternative possibility is that the effects we have observed are largely due to variations in the energy spectra and pitch-angle distributions of precipitating electrons associated with the substorm. Indeed, intense spikes of energetic electrons with pitch-angles almost parallel to the local magnetic field direction have recently been observed by the polar orbiting satellite OGO-6 (ref. 16). If, however, the bulk of the electrons involved have energies which are not greatly in excess of ~ 400 keV it is difficult to explain the difference between the relatively intense absorption observed at 51.4 MHz in comparison with that at 30 MHz. Of course some combination of these suggestions may be required to give a fully satisfactory explanation of the observations.

The absorption spikes have so far only been observed during the evening hours (1900–2200 CMLT). This result is consistent with statistical studies of riometer absorption¹⁵, which reveal one maximum of occurrence in the evening hours and with the pattern of occurrence of particle spikes observed by satellites¹¹. Auroral observations made from ISIS-2 also suggest that the initial brightening of substorms occurs more frequently in the late evening sector than in the midnight sector¹⁴.

We plan to extend the scope of these observations by making suitable changes in the system. A second broad-beam 51.4-MHz system is being set up in order that a definitive distinction can be made between spectral and spatial effects. We also intend to make photometer and television observations to see whether or not similar phenomena occur in the optical emissions. Simultaneous observations will be made using the VHF radar system 'STARE' which was recently put into operation¹⁷, and correlations will be made with energetic particle observations from the satellite GEOS.

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A proposed cadmium-selenium film-electrolyte Schottky barrier solar cell

ECONOMIC analyses of photovoltaic solar cells show that a considerable manufacturing cost reduction will be required before such devices can give a positive contribution to the World's energy needs. To this end, efforts have been made to divert some research work from the high-quality single-crystal

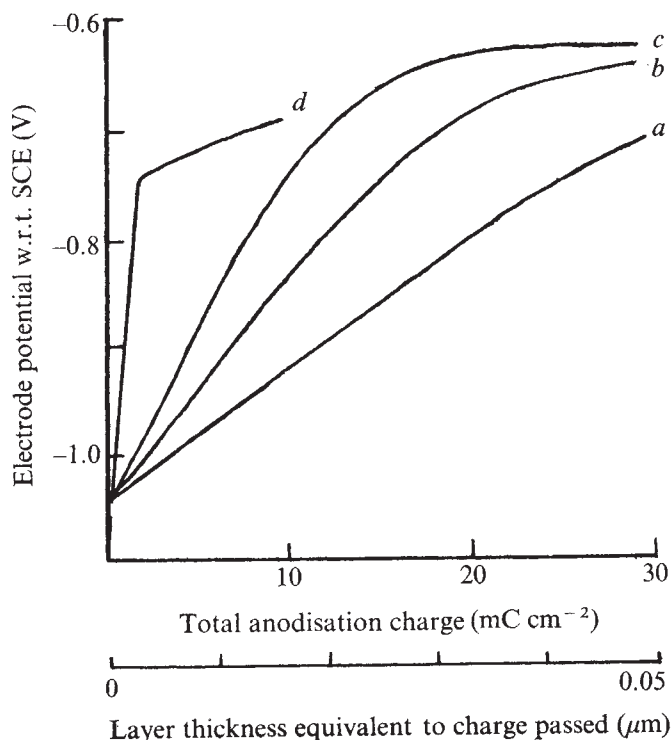
technology, which supports the present commercial solar cells, to alternative systems incorporating less pure, polycrystalline materials, deposited as thin layers to avoid wastage of expensive elements. This letter describes the operation of a novel system which may be constructed from relatively inexpensive materials, yet which converts solar radiation into electrical energy with an efficiency greater than some similar systems previously reported.

Considering the replacement of single crystals by polycrystalline materials, we find that grain boundaries can contribute to the following major limitations to the ultimate cell efficiency: first, recombination of photogenerated electron-hole pairs occurs if the optical absorption distance exceeds the minority carrier diffusion length, which is limited by the grain size; secondly, the formation of a well-defined p-n junction is complicated because of preferential adsorption of dopants at grain boundaries. The former may be avoided by the use of direct band-gap semiconductors, and the latter by replacement of the p-n junction with a Schottky barrier.

A recent development in Schottky barrier cells was the semiconductor-electrolyte liquid junction in which the usual metallic contact was replaced by a suitable electrolyte solution¹. Ellis *et al.*², showed the systems CdS (single crystal)-Na₂S_n solution and CdSe (single crystal)-Na₂S_n solution to be stable under illumination, in contrast to previous systems which had degraded by photochemical reactions. A similar electrolyte was seen to preserve semiconductor stability when Miller *et al.*³ constructed solar cells using anodically prepared films of CdS and Bi₂S₃. The efficiency of the single crystal CdS cell was limited by the high band-gap of CdS whereas CdSe gave a remarkable efficiency when used in etched single crystal form. Anodic Bi₂S₃, however, was found to be less efficient than anodic CdS despite its favourable band-gap. Since CdSe has been shown to be more efficient than CdS in single crystal form, it was thought that a comparison of the respective polycrystalline films would be informative.

Anodic films of CdS were prepared in constant current conditions. The Cd anode was kept in the dark and the elec-

Fig. 1 Galvanostatic anodisation of Cd in sulphide and selenide solutions. *a*, 1 M Na₂S, 2 μ A cm⁻²; *b*, 1 M Na₂S, 5 μ A cm⁻²; *c*, 1 M Na₂S, 10 μ A cm⁻²; *d*, 1 M NaOH + Se, 2 μ A cm⁻².



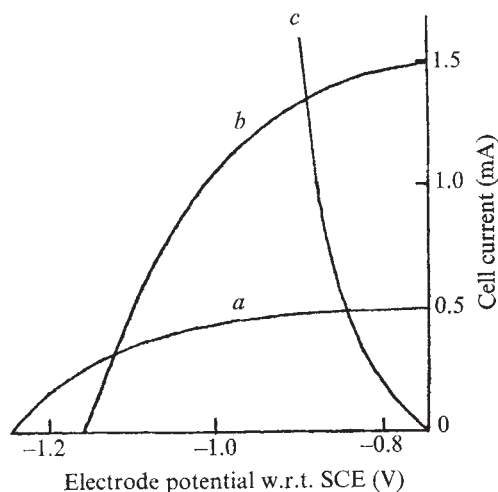


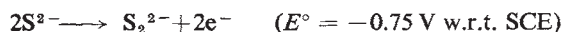
Fig. 2 Current potential characteristics: *a*, Illuminated CdS layer (0.5 cm²) in molar Na₂S, 0.05 mol dm⁻³ S solution, *b*, Illuminated CdSe layer (0.5 cm²) in 1 M Na₂S, 0.05 mol dm⁻³ S solution, *c*, 10 cm² Pt counterelectrode in molar Na₂S, 0.05 mol dm⁻³ S solution.

trolyte, molar Na₂S, was continuously purged with argon. Fig. 1 shows the anode potential with respect to a saturated calomel electrode (SCE) as a function of charge passed. The initial potential shows that the reaction was self-driving according to the equations

(for example) $\text{Cd} + \text{S}^{2-} \rightarrow \text{CdS} + 2\text{e}^-$ $E \approx -1.06$ V at anode

and $\text{e}^- + \text{H}_2\text{S} \rightarrow \text{HS}^- + \frac{1}{2}\text{H}_2$ $E \approx -0.75$ V at platinum cathode

As the CdS film grew, the anode potential rose corresponding to the field required to assist diffusion of Cd²⁺ or S²⁻ ions through the CdS lattice. The potential did not rise above -0.6 V, suggesting that some current was thereafter being diverted to a competitive reaction, for example



The thickness of the film can be estimated by applying Faraday's law to the integrated current. We conclude that growth becomes very slow after about 0.1 μm. Anodisation of Cd in molar Na₂S, S at 0.5 mol dm⁻³ solution gave similar results. Fig. 1 also shows the result of anodising Cd in molar NaOH saturated with selenium—a solution known to contain polyselenide and selenide ions. This time we see that the competitive reaction occurred much earlier, so that thicknesses of above 0.01 μm were difficult to achieve by this method. Since a thicker layer would be required to efficiently absorb light, alternative methods of preparation were investigated. One method was the immersion of Cd in a boiling solution of KOH at 5 mol dm⁻³ saturated with selenium. After some hours treatment, a black layer was revealed on the Cd surface after removal of selenium in boiling Na₂S solution. We presumed this layer was CdSe.

Figure 2 shows cell current plotted against electrode potential for the cadmium sulphide and selenide films illuminated by direct sunlight in a solution of molar Na₂S, 0.05 mol dm⁻³ S. The potential was measured against an SCE immersed in the solution while the current passed through a platinum counterelectrode. In this way the potential of the photovoltaic electrode was observed undistorted by any overvoltage developed on the platinum. Approximate conversion efficiencies, assuming no overvoltage on the counterelectrode, were 0.4% for CdS and 1.0% for CdSe.

We have shown that films of CdSe may be prepared by an economically attractive process, involving no high temperatures and using a minimum amount of the less abundant element, selenium. The films were shown to have a greater efficiency than similar sulphide films as expected from band-gap considerations. Further work involving alternative preparations

of the films is showing efficiencies significantly greater than that reported here, and stability tests have as yet shown no signs of film degradation.

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Iceberg deterioration

A VERY large tabular iceberg was observed as it drifted north-east of the Grand Banks of Newfoundland during May and June 1976. When the iceberg was first sighted on 12 May 1976 it showed only minor signs of deterioration. From 12 May until last sighted on 6 June the iceberg underwent a rapid reduction of the above water surface area with the erosion largely confined to the turbulent layer associated with gravity waves. The erosion progressed along lines parallel to the structure of the iceberg as indicated by the pronounced ridge pair seen in the photographs (Fig. 1), a trellis drainage pattern, and an alternation of light and dark bands over the entire surface of the iceberg.

Photographs were taken of a large number of icebergs during the 1976 flights of the International Ice Patrol, operated by the US Coast Guard. These photographs are to be used for a study of iceberg populations off the Grand Banks of Newfoundland (Fig. 2). Black and white photographs (9-inch format) were taken from altitudes of between 300 and 3500 m.

Among the photographs obtained are a unique series of five taken of the same iceberg over a period of 25 d (Fig. 1). It is highly unusual for an iceberg to be relocated over such an extended period when positive identification is possible. Icebergs normally change their appearance so radically by a combination of calving, melting and rolling that it is impossible to positively identify them after only a few days. In this case the unusually low profile, only 4-5 m of elevation, and tabular shape maintained the iceberg in an extremely stable condition.

From 12 May to 6 June 1976 the iceberg decreased in surface area from approximately 190,000 m² to an area of 109,000 m². The rate of decrease in surface area was nearly linear (Fig. 3) and resulted from wave erosion, undercutting and minor calving. The surface water temperature was 2-4 °C. Sub surface temperatures in this area typically decrease to a minimum of less than -1 °C at 75-100 m depth.

Wave erosion was concentrated at those points which appear as slight irregularities in the 12 May 1976 photograph. The progressive enlargement of these embayments was the result of the local concentration of wave energy and continued bathing of the ice by the turbulent water. The embayments seemed to extend only several metres below the water's surface and have an orientation parallel to the structural features of the iceberg. The underwater shape of the iceberg is not known but it is suspected to have had a flat bottom as inferred from the long term maintenance of its top parallel to the sea surface.

The iceberg seemed to be of land ice origin. Analysis of a piece of the iceberg, recovered by the USCGC Evergreen, showed it to be fresh water ice. The characteristic air bubble bands of glacier ice were visible and surface melt tests produced a pattern of hexagonal depressions

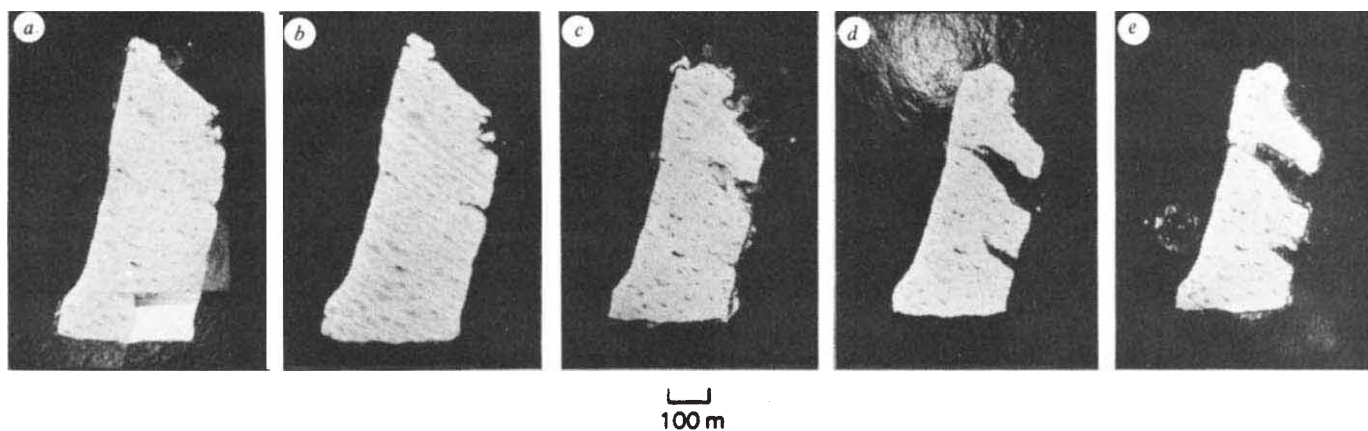


Fig. 1 The deterioration of an iceberg showing the progressive enlargement of embayments. *a*, Photograph 1708Z, 12 May 1976. 49°19.9'N, 49°33.3'W. Area 190,485 m². *b*, 1804Z, 13 May 1976. 49°28.9'N, 49°26.6'W. Area 189,126 m². *c*, 1404Z, 31 May 1976. 48°09'N, 49°34'W. Area 137,361 m². *d*, 1343Z, 4 June 1976. 47°39.7'N, 49°15.8'W. Area 121,113 m². *e*, 1812Z, 6 June 1976. 47°31'N, 49°07'W. Area 109,067 m². Scale bar, 100 m.

approximately 2 cm across which typify the crystalline melt surface texture of glacier ice. The two linear parallel ridges in the upper third of each picture of Fig. 1 are the most obvious manifestations of the ice structure. In the original photographic prints a pattern of trellis drainage can be seen which indicates structural control. There is also an alternation of light and dark bands which while quite subtle are obvious on close examination of an original photographic print. The banding is not a result of the drainage pattern since it is crossed by drainage channels in numerous locations. The band pairs do not seem to be regularly spaced nor are they perfectly linear or parallel for the width of the iceberg. We feel that such a structure is likely to be the result of flow and that the light and dark bands represent streamlines. The two parallel ridges are possibly the result of shear or flow over an irregularity of the glacial bed.

The iceberg's origin remains unknown. The Ward Hunt ice shelf on northern Ellesmere Island, Petermann Glacier in Hall Basin and Humboldt Glacier in Kane Basin are all capable of producing a thin tabular iceberg. Usually such icebergs fragment and deteriorate before they reach 50°N latitude. The last sighting of a similarly-shaped iceberg on

the Grand Banks by the International Ice Patrol was in 1964 (ref. 1). Twenty large tabular icebergs, some as long as 600 m, were sighted by the Ice Patrol aircraft in late February of 1964 between Hamilton Inlet and Cape Chidley. They appeared as far south as 44°N by early May 1964. These icebergs were thought to have their origin in ice island WH-5 which was observed to block the Kennedy Channel from the Canadian shore to Hans Island in 1963 (ref. 2).

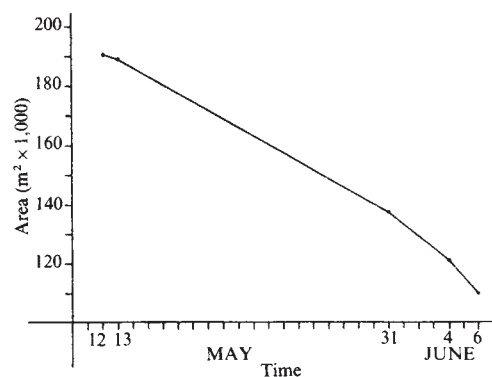


Fig. 2 Drift track of a large tabular iceberg near the Grand Banks of Newfoundland.

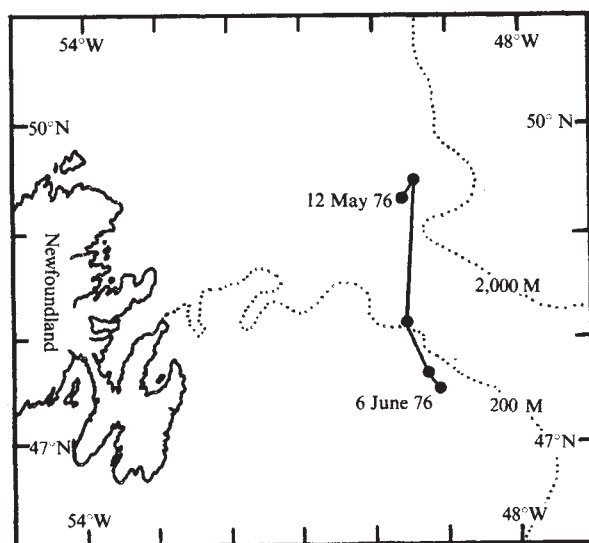


Fig. 3 The reduction of the sea level horizontal cross-sectional area as a function of time.

A low tabular berg of this size is quite unusual and fortunately rare. It represents a greater than usual hazard for surface vessels due to its lower probability of detection particularly in a seaway. The iceberg's uniqueness contributed to the attention given it by the International Ice Patrol.

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Upwelling by icebergs

A CASE is made for considering the influence of melting icebergs on the oceanographic characteristics of the Weddell Sea. In particular, the equivalent upwelling produced by melting must play a large factor in the supply of nutrients to the surface layers of this ocean. It might be, for example, that the slowly moving icebergs leave behind a trail of nutrient enhanced surface fluid. The need for testing such a hypothesis is clear.

In Antarctica the net annual yield of icebergs from the ice shelves and direct glacier streams is of order 10^{15} kg (ref. 1). The major shelf regions are the Ross, Amery and Ronne-Filchner. The latter supplies icebergs to the Weddell Sea at an annual rate of about 0.12×10^{15} kg; a similar amount is estimated to enter this sea from direct glacier streams and smaller shelves. The average density of the ice is 0.85 g cm^{-3} (ref. 2) and its temperature near the edge of the shelf of origin is of order -10°C (ref. 3).

Gordienko⁴ produced a sampling of the size distributions of icebergs off the coast of East Antarctica near the Amery ice shelf. His data show that one-half of the individual icebergs have standing heights above water of from 28 to 65 m, with frequency uniformly distributed between these limits. Average length of iceberg is about 1 km (ref. 4) although individual bergs have been reported with lengths of 45 and 70 km (ref. 5). Total iceberg accumulation in the Antarctic Oceans has been estimated at 6×10^{15} kg, which implies an average iceberg lifetime of about 6 years (ref. 1).

Studies have been reported on the economic usefulness of transporting Antarctic icebergs to lower latitudes as sources of potable water (refs 1 and 6), but no study has been reported on the role that the icebergs have in modifying the structure of the oceans in which they melt. Surface circulation in the Weddell Sea is part of a large clockwise gyre; hence the region tends to retain icebergs and the potential for modification of its near-surface structure of temperature and salinity by the icebergs is greater than that for other Antarctic locations. The results given here show that the icebergs have a significant role in causing the upwelling of waters from below the pycnocline into the shallow upper layer, and that a significant amount of buoyant potential energy is released into the upper layers of the ocean by the melt process.

The vertical structure of the upper waters of the Weddell Sea are typically as shown in Fig. 1 where the temperature and σ_t data for three stations from the 1973 Glacier cruise⁷ are given. The base of the pycnocline is at about 50 m depth. Below the pycnocline is Atlantic Warm Deep Water. An iceberg of 45 m height above water (the modal value of the Gordienko distribution) would extend some 200 m below the pycnocline and its melt water and heat sink properties would be available for modification of this water. A two-layer approximation to the representative structure shown in Fig. 1, averaged over all of the Glacier data for the Weddell Sea in Jan-Feb 1973 would be as shown in Table 1.

The potential energy released by melting below the pycnocline, and thus available for mixing between the two layers can be estimated. We assume that the dominant melting rate occurs along the sides of the iceberg. The potential energy released below the pycnocline is

$$\text{P.E.} = 0.85 \int_{25,000}^{5,000} \Delta \rho g z dz = 6.4 \times 10^9 \text{ erg per cm}^2 \text{ ice column}$$

where $\Delta \rho$ is the density difference between melt and ambient water and is of the order $25 \times 10^{-3} \text{ g cm}^{-3}$.

If melting along the bottom of the iceberg is the dominant rate, the potential energy released below the pycnocline is slightly greater than calculated above since the iceberg will sink

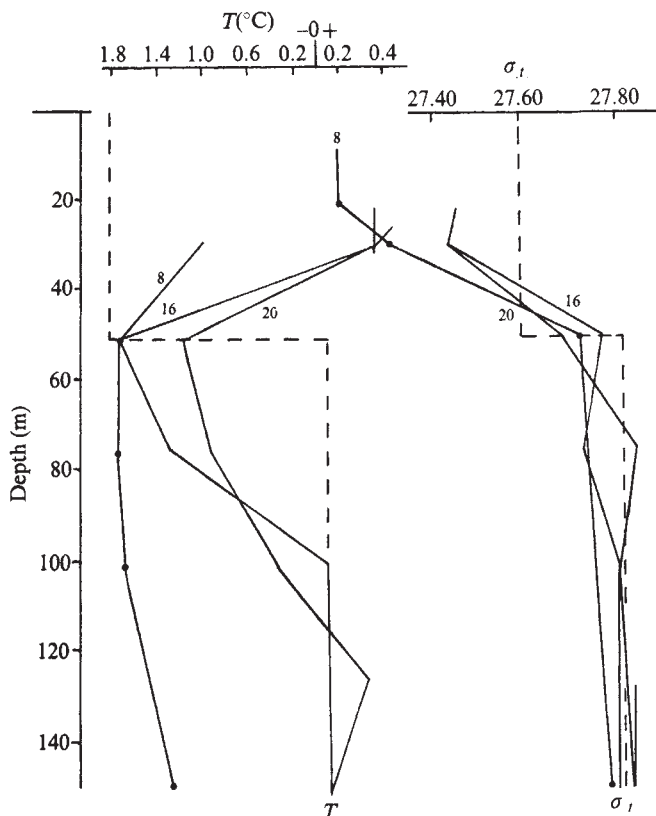


Fig. 1 Representative temperature and density structure of upper waters of the Weddell Sea⁷. Stations are located as follows: station 8: $63^\circ 26' \text{S}$, $26^\circ 45' \text{W}$; station 16: $68^\circ 50' \text{S}$, $18^\circ 20' \text{W}$; station 20: $70^\circ 10' \text{S}$, $15^\circ 10' \text{W}$. Dashed lines show a two-layer approximation based on an average of all 1973 Glacier stations in the Weddell Sea.

as the bottom melts and hence more ice melts below the pycnocline than in the case of melting along sides only. The opposite holds if melting above the water line is the dominant rate.

It is worth while to compare this energy release within the lower layer with that transferred from wind stress at the surface. The 1.2×10^{14} kg of icebergs produced annually by the Ronne-Filchner shelf alone represent $4.8 \times 10^{13} \text{ cm}^2$ of mode thickness bergs. Total energy release is then $3.1 \times 10^{22} \text{ erg yr}^{-1}$ or about $10^{15} \text{ erg s}^{-1}$. A usual wind stress figure is 1 dyn cm^{-2} . Niiler⁸ has shown that the wind related energy which passes through the mixed layer is then about $1 \text{ erg cm}^{-2} \text{ s}^{-1}$. Thus, the energy input by iceberg melt is roughly equivalent to the average wind stress input over an area of 10^6 km^2 .

Consider an iceberg of density 0.85 g cm^{-3} , area of 1 km^2 and extending 250 m into the water. That portion of the iceberg extending below the pycnocline contains $1.7 \times 10^8 \text{ m}^3$ of melt water. What is the ultimate disposition of this melt water?

We assume first that all ice melt mixes with surrounding lower layer fluid to produce a fluid of density equal to that of surface water, that is, water at 0°C , 34.00‰ and that all mixture water ascends to the surface. Let V_1 be the volume of 34.57‰ water

Table 1 Two-layer approximation to a representative temperature and density structure of the Weddell Sea averaged over all of the Glacier data for Jan.-Feb. 1973

Upper layer Thickness 50 m		Below pycnocline	
At 20 m		At 150 m	
Temperature	-1.8 to 0.6°C	Temperature	0.2°C
Salinity	33.94% $\left\{ \begin{array}{l} +0.29\% \\ -0.55\% \end{array} \right.$	Salinity	34.57%

required by the mixture to attain density equal to that of surface water, and V_2 the resultant volume of the mixture. Then

$$V_2 - V_1 = 1.7 \times 10^8$$

and

$$34.57 V_1 = 34.00 V_2$$

Solving for V_2 , one obtains 10^{10} m^3 of mixture water of density equal to that of surface water, for each square kilometre of iceberg. The vertical migration time of the mixture will be short relative to the melt time of the iceberg. Hence, if the iceberg melts uniformly within one year, an average vertical volume transport of mixture water into the upper layer would be of the order $3.3 \times 10^8 \text{ cm}^3 \text{ s}^{-1}$.

Vertical velocities found in the Oregon Coastal Upwelling zone are of the order $10^{-2} \text{ cm s}^{-1}$ (ref. 9). Thus the area of coastal upwelling required to produce an annual vertical transport equivalent to that of the 1 km^2 iceberg is 3.3×10^{10} or 3.3 km^2 .

Since the iceberg presumably melts at a uniform rate throughout the year, whereas Oregon upwelling is effective only 3 months each year, the coastal upwelling equivalent area may again be increased by a factor of four, to a value of 13.2 km^2 per each square kilometre of iceberg.

In contrast to coastal upwelling, iceberg upwelling is permanent. In Oregon waters, for example, some fraction of the nutrients which are contained in the upwelled water are returned to the deeper zones unused at the time of subsequent sinking of the upwelled waters at the frontal zone. We estimate a 50% utilisation of nutrients in the Oregon case (Lawrence F. Small, personal communication). Then one must again increase the coastal upwelling equivalent area by a factor of two, to 26.3 km^2 .

For an annual iceberg production in the Weddell Sea region of $1.2 \times 10^{14} \text{ kg}$, or $1.2 \times 10^{11} \text{ m}^3$, the number of square kilometres of mode sized icebergs produced and melted each year is 1,400. In terms of area of upwelling equivalent to that of Oregon coastal upwelling, the Weddell Sea has $1,400 \times 26.3$ or $36,800 \text{ km}^2$ of such area. Stated another way, since the Oregon coastal upwelling region extends some 10 km to sea, the upwelling produced in the Weddell Sea by melting icebergs is equivalent to some 3,700 km of Oregon coastline, a distance approximately equal to the length of coastline of the North American continent from Acapulco, Mexico to Ketchikan, Alaska.

The importance, as shown above, of upwelling by icebergs in Antarctica is striking, and one immediately returns to question the validity of the underlying assumptions. Two of the assumptions are critical: first, that melt water entrains sufficient ambient fluid to acquire a density equal to that of fluid in the surface layer, and second, that all of the mixture reaches the surface layer. A key element in evaluating the validity is to examine the Grashoff number which characterises a free convection regime along a vertical wall at which buoyant energy is generated.

For a vertical, heated wall in air, the Grashoff number is given by¹⁰

$$N_{Gr} = \frac{l^3 \rho^2}{\mu^2} g \beta (T_f - T)$$

where l is the characteristic vertical length (the depth of the iceberg below the pycnocline), ρ is the density, g is gravity, β is the thermal expansion coefficient, T_f is the fluid temperature in the field far from the wall, T is the wall temperature, and μ is the fluid viscosity. In the case of iceberg melt, the Grashoff number is predominantly affected by the density difference between that of melt water, ρ , and that of surrounding saline sea water, ρ_f : hence

$$N_{Gr} = \frac{l^3 \rho^2 g}{\mu^2} \left(\frac{1}{\rho} (\rho_f - \rho) \right)$$

For a density difference ($\rho_f - \rho$) of 0.025 g cm^{-3} , one obtains a Grashoff number of the order of 10^{17} . This is an extremely high value which places the vertical flow along the sides of the melting iceberg clearly within a fully turbulent state. For this reason, it is reasonable to assume the conditions stated above.

An iceberg with a plane bottom coincident with a level surface will not cause free vertical convection since the melt fluid along the bottom is constrained from rising by the iceberg itself. If the bottom is tilted relative to a level surface, melt fluid will experience vertical acceleration although the Grashoff number would be smaller than that which characterises flow along vertical sides. Clearly, some bottom melting must occur, hence the released energy estimation previously stated is conservative.

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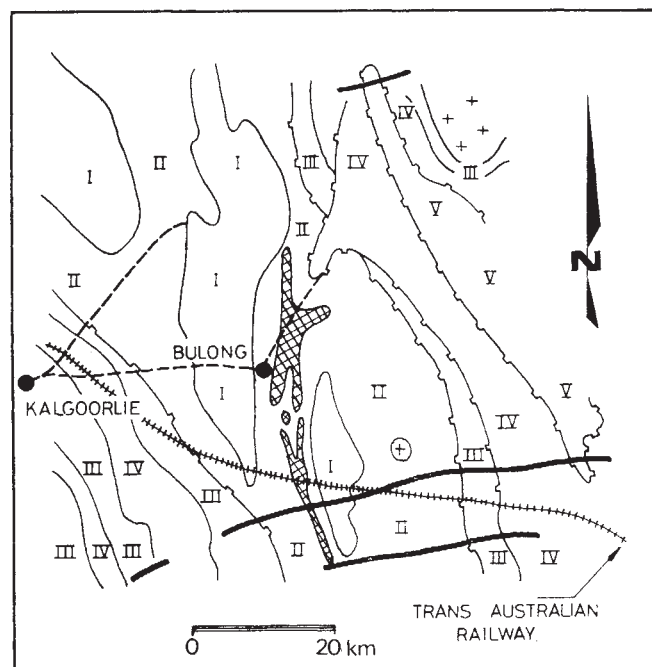
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New type of igneous layering due to fractionation of Archaean ultramafic magma

RESEARCH into the petrogenesis of the Bulong Complex¹, near Kalgoorlie, Western Australia (Fig. 1), has indicated that it is composed largely of layered ultramafic cumulates which exhibit both phase and cryptic layering indicative of low pressure fractionation of Archaean ultramafic magma.

The Bulong Complex covers some 200 km^2 but its greatest stratigraphic thickness is only 1.1 km. Detailed research¹ has indicated that the complex originated as a series of liquid-rich, ultramafic magma pulses which were introduced, largely concordantly, into an extensive, but narrow, stratigraphic zone of the originally flat-lying, host formation.

Fig. 1 Geological setting of the Bulong Complex (hatched areas).



Smaller ultramafic pulses either froze to form homogeneous olivine-rich, 'quench-texture' sills or underwent flowage differentiation, before freezing, to form crudely zoned ultramafic sills with olivine-rich cores. Larger ultramafic pulses, on the other hand, differentiated *in situ*, largely by gravitational processes, to produce layered olivine-rich sills which are capped in part by layered pyroxene and pyroxene-plagioclase bearing horizons. During fractionation, small pulses of derivative ultramafic to acid magma were probably tapped off at various stages and intruded about the top of the complex to form the numerous small sills and dykes which are now observed in that position. When igneous activity had virtually ceased the complex and its host rocks were regionally folded (three periods of variably tight to open folding are evident and the complex is even locally overturned) and metamorphosed to middle greenschist grade^{1,3}.

Layered, olivine-rich sills constitute most of the complex and in most localities 3 to 5 sills, stacked one on top of the other and separated in part by thin sheets of country rock, are evident. The sills mainly range in thickness from 350 to 400 m and although all have been deformed, serpentinised and regionally metamorphosed, primary textural features are well preserved and primary mineral relics are not uncommon. The least altered sills contain thin (1–3 m thick) remnant basal 'chilled border' zones composed of olivine-rich, porphyritic spinifex rock⁴ (the presumed quenched starting liquid)¹. About 95%, or more, of the overlying layered rocks are olivine cumulates, which contain accessory amounts of cumulus chromite, in which the proportion of orthocumulus material increases gradually from about 5%, or less, in lowermost parts to 30%, or more, in uppermost parts. The orthocumulus minerals in decreasing order of abundance, are clinopyroxene, orthopyroxene and plagioclase, although hornblende and biotite also occur as orthocumulus phases near the tops of some sills. The thick, layered olivine-(chromite) cumulate zones are capped in part by thin orthopyroxene cumulate layers which are in turn capped successively by thin orthopyroxene-clinopyroxene and plagioclase-clinopyroxene-orthopyroxene cumulate layers. In at least one of the sills a thin chromitite (chromite cumulate) layer is located directly beneath the orthopyroxene cumulate layer. All sills exhibit normal cryptic layering delineated by slight, but consistent iron enrichment in relict olivine (Fo 93 to Fo 86), chromite ($\Sigma\text{FeO}=18$ to 36 wt%; $a_0=8.26$ to 8.31 Å) and clinopyroxene ($\text{Ca}_{15}\text{Mg}_{51}\text{Fe}_4$ to $\text{Ca}_{42}\text{Mg}_{49}\text{Fe}_9$) with increasing stratigraphic height. Clinopyroxene occurs mainly as an intercumulus phase but topmost clinopyroxenes are cumulus and co-exist both with cumulus orthopyroxene (En15) and cumulus plagioclase (An 80 to An 85). Within the main layered olivine-(chromite) cumulate zones there is a marked upward increase in whole rock Ti (1,000–3,000 p.p.m.) and P (50–200+ p.p.m.) content and a marked decrease in Ni (3,500–1,000 p.p.m.) content. This is largely a reflection of the gradual upward increase in the proportion of orthocumulus material¹.

The field evidence^{1,2} suggests that at the time of emplacement of the layered sills the confining pressure due to overlying rocks was only about 1 to 2 kbar and this is supported by (1) high CaO content (0.3–0.5 wt%) in relict olivine^{5,6}, (2) moderate to high $\text{Al}^{\text{IV}}/\text{Al}^{\text{VI}}$ (>2) ratios⁷ and TiO_2 contents (0.2–0.6%) (ref. 8) in relict clinopyroxene, (3) abundant evidence for reaction between settled olivine and trapped liquid⁹ before onset of intercumulus pyroxene crystallisation and (4) by a general lack of evidence for subsolidus reaction between cumulus olivine and intercumulus plagioclase¹⁰. Furthermore, simplified low-pressure (1–2 kbar) phase relations for the system olivine-clinopyroxene-plagioclase-quartz¹¹ accurately predict the observed crystallisation sequence obtained using the mean composition of the basal, chilled contact rocks¹. The one

known chromite horizon is thought to have simply formed as a 'lag deposit', partly because of the peritectic relation between orthopyroxene and chromite¹² (that is, orthopyroxene precipitation suppresses chromite precipitation) and because of the relative settling rates of olivine (coarse-grained) and chromite (very fine-grained)¹⁴.

Liquidus phase relations for natural ultramafic rock of similar composition to that of the chilled contact rock (Table 1)¹³ clearly indicate that the ultramafic magma pulses which gave rise to the Bulong Complex were low in water content (1–2 wt%) and were also emplaced at temperatures slightly in excess of 1,650 °C. Finally, the TiO_2 content of the mean chilled contact rock (Table 1) suggests that it was derived by approximately 60% partial melting of an Archaean upper mantle source¹⁵.

Table 1 Chemical analyses of ultramafic quench rocks (recalculated anhydrous)

	Ultramafic quench rock from South African Archaean flow ¹³	Mean basal chilled contact rock in Bulong Complex (5 analyses)
	a	b
SiO_2	46.5	43.4
TiO_2	0.2	~0.25
Al_2O_3	3.6	4.5
ΣFeO	10.3	7.0
MgO	33.0	39.0
CaO	5.0	3.1
Na_2O	0.5	0.4
K_2O	0.2	0.2
Cr_2O_3	0.4	0.4
NiO	0.3	0.3
$\text{CaO}/\text{Al}_2\text{O}_3$	1.4	0.7

Discovery of the phase and cryptic layering reported here has not only extended our knowledge of the spectrum of Archaean ultramafic rock types, which up until now has included various types of zoned and unzoned non-layered sills and flows (refs 4, 15–17), but has also broadened our knowledge of the total spectrum of layered igneous rocks¹⁸. A detailed account of phase and cryptic layering in the Bulong Complex is in preparation.

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Effects on biological evolution of changes in ocean chemistry

PERHAPS the most perplexing problem of biological evolution involves explanation of why diversification of life began only after 85% of the lifetime of the Earth while simple life seems to have been present for virtually all of geological time. Recent observations¹⁻² on the presence of complex organic species in meteoritic debris and in the interstellar medium show that some of the basic biological building blocks may have been available from the earliest times. Chamberlain and Marland³ have commented on the biological consequences of the Hargraves⁴ model of ocean-crust evolution while others⁵⁻⁶ have reassessed evidence on atmospheric evolution and indicated that changes in oxygen partial pressure may not have been critical to the diversification of life. In this note I suggest that important changes in ocean chemistry must have occurred if our current models of crustal evolution and igneous events have any validity. The evolution of complex life forms requires a high degree of environmental stability and constancy of chemical environment. It is suggested that this condition is closely linked to the spacing of major volcanic centres, and that the modern long wavelength pattern of ocean ridges and subduction zones was necessary to achieve this stability. The distribution of ophiolites or ocean-floor structures of modern type⁷ indicates that the present pattern may have been initiated about one billion years ago. Furthermore, large scale continental emergence⁸ may have been necessary to provide oceans with stable chemistry.

There is much evidence to support the Hargraves⁴ model of early globe-encircling oceans and slow continental emergence. The ocean mass may have been greater⁸. Further evidence shows that with an early hotter earth, volcanic activity was more intense and that centres of volcanic activity were closely spaced with a wavelength pattern of hundreds not thousands of kilometres. We also know that the cooling of submarine igneous bodies involves convective flow of seawater and that the chemistry of the cold inflow and hotter discharge waters is quite different. Thus, early oceans would tend to reflect volcanogenic discharge more than continental runoff. As such, chemistry would be erratic and frequently concentrations of typical easily transported metals like Cu, Hg, Pb, Zn, Mn, Fe, As, could be large as in the modern Red Sea deeps. (The Red Sea illustrates⁹ the toxic effects of such discharge.)

For the present earth, continental runoff amounts to about 3×10^{19} g yr⁻¹ while the ocean mass is 1.4×10^{24} g. Submarine volcanic activity associated with the ocean-floor spreading process currently adds some 3×10^{16} g of basalt liquids to the ocean annually. This liquid at 1,200 °C cools by convective flow of ocean water. Sites of discharge will cause local changes in chemistry as seen in the Red Sea and many other places^{10,11}. The energy input at modern ocean ridges (4×10^{19} J yr⁻¹) could heat 10^{17} g of sea water to 100 °C. Thus, at present, hydrothermally modified ocean water might approach 1% of the volume of continental runoff. If this water contains any chemical species at much higher concentrations than in river water, it may influence the balance of ocean waters.

Elements that are present at much higher levels than in normal ground waters (100 times) include a host of those which can be toxic to complex life forms (As, Hg, Cu, Zn, Mn, Fe, Pb). Furthermore, if there was little land above sea level in the Archaean, the input of metal absorbing clay mineral would be reduced, and this detoxification process would be less effective¹².

At present, while basaltic mantle-derived volcanic processes are concentrated in the submarine environment, subduction andesite-granite igneous activity is concentrated

on the continents and if convective cooling occurs, it involves less saline waters which in general transport less metal. But in the Archaean, nearly all igneous activity was submarine. The huge granite bodies of the Archaean could generate sustained submarine discharge on a scale not seen today. Imagine the consequences of emplacing the Sierra Nevada Batholith in a submarine environment.

Thus, the Archaean ocean floor may have been populated by closely spaced volcanic centres with an igneous intensity an order of magnitude more than known today¹³. From such centres there would have been an intense discharge of toxic elements and hydrogen into deep basins. In such an environment, one would have expected only the most simple and versatile life forms to survive.

Runcorn¹⁴ has discussed the possibility of quantised changes in mantle convection associated with cooling. If cells have become larger with time, then the current convection pattern may have been necessary for the recent biological diversifications. Fluctuating environmental pollution caused by closely spaced centres of intense submarine volcanism may have inhibited complex biological evolution for most of earth history.

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Interaction between effects of vesicular-arbuscular mycorrhiza and fertiliser phosphorus on yields of potatoes in the field

MANY experiments in controlled environments have shown that vesicular-arbuscular (VA) mycorrhizas may benefit the phosphorus uptake and growth of plants¹⁻⁴, depending on the nature and level of indigenous inoculum, the phosphorus status of the soil and other factors⁵⁻⁷. But there have been few reports of attempts to obtain larger crops, or reduce phosphorus fertiliser requirement, by manipulation of VA mycorrhizal infection in field crops⁸⁻¹². We report here the effect on potatoes of an artificial increase in the level of VA infection. Potatoes are known to have VA mycorrhizas¹³⁻¹⁶, and their sparse rooting systems¹⁷ and heavy phosphorus demand suggest that they should respond to infection. This is the first report of a successful field inoculation experiment on a major field crop in temperate zone conditions.

The method of inoculation depended on observations, made in a separate experiment, on the changes of spore number and extent of infection caused by different cropping sequences. Cropping with cereals tended to induce high spore density, with heavy infection of subsequent crops, whereas if the area was left fallow there was a rapid decline in spore density and subsequent level of infection (Table 1).

The potato experiment was sited on an area left fallow for 2 yr, so that the spore density was only about one per ml. The inoculum consisted of topsoil from nearby plots,

Table 1 Effect of crop sequence on VA mycorrhizas in barley, together with spore density in soil

Growing season		Infection in August (% of root length)	Spore density (no. per ml of soil, fraction retained on 45-µm sieve)	
			Spring	Autumn
1975	Continuous barley (4 yr)	39	29	21
	Barley after 1 yr fallow	21	18	10
1976	Continuous barley (5 yr)	19	17	17
	Barley after 2 yr fallow	5	4	Not measured

on the same soil series, on which barley had been grown for 3 yr, and which contained 13 spores per ml. The most frequent spore type was identified as *Glomus macrocarpus*¹⁸. The experimental plots were 3.05 m × 3 m, and received ammonium nitrate (437 kg ha⁻¹) and potassium chloride (502 kg ha⁻¹) fertiliser broadcast before planting of Pentland Javelin seed potatoes. They were treated with triple superphosphate (0 or 481 kg ha⁻¹), combined factorially with presence or absence of soil inoculum. The latter was applied down the furrows at about 3 kg m⁻². It contained available (Olsen) phosphorus at 15 µg g⁻¹, compared with 7 µg g⁻¹ in the plot soil; the amount of added available phosphorus was therefore extremely small compared with any useful fertiliser dressing. Uninoculated plots received the same amount of topsoil from the fallowed area. There were four replicates. During the period of growth, soil core samples were taken, the roots in them were washed out and percentage infection was determined¹⁹.

Infection spread most between the first two root samplings (Table 2). Infection with soil inoculum was much greater than the very low control level but, as expected, phosphorus fertiliser suppressed it considerably. No treatment had a significant effect on phosphorus in leaflets sampled on 12 July (Table 2), possibly because leaf senescence was becoming general.

At harvest, fertiliser phosphorus increased yield, but least so in the presence of cereal soil inoculum. Interaction between inoculum and fertiliser phosphorus is indicated by the large increase in yield caused by inoculum, but only in the absence of phosphorus fertiliser.

An important purpose of our trial was to test the effectiveness of a relatively small amount of soil as inoculum for VA mycorrhizal infection. Rich and Bird¹⁰ reported successful use of imported soil containing twice as many spores as the soil at the experimental site, whereas our soil inoculum had more than 10 times as many spores as the field soil, but was used in smaller quantities. The micro-

biological population of the inoculum soil and the plot soil must have differed in many respects, including their content of pathogenic organisms, because of their different cropping history. Other organisms in the inoculum soil may have infected or altered the crop, but the increase in mycorrhizal infection, and the interaction with phosphorus fertiliser, leaves little doubt that it was principally VA organisms which affected the yield.

Our experiments illustrate two important points. First, the effects of cropping rotation on the VA infection of subsequent crops must always be considered²⁰. Second, the phosphorus status, fertiliser response and economics of the use of fertiliser for mycorrhiza-forming field crops may depend greatly on the degree of infection, and work on these may be incomplete unless infection is measured. The frequent difficulty of correlating phosphorus requirement of a crop and soil analytical results may also be due to differences in VA infection.

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Chemical activation of host defence mechanisms as a basis for crop protection

THE possibility that a chemical for plant disease control might function by activating the natural resistance mechanisms of the host has been suggested previously¹, but there are few well-documented examples. We report here that 2,2-dichloro-3,3-dimethyl cyclopropane carboxylic acid (WL 28325) may exert its systemic fungicidal activity against the rice blast disease (caused by the fungus *Piricularia oryzae*) in this way.

It is unlikely that the WL 28325-induced resistance of rice to blast can be attributed to either the direct action of the fungicide on the parasite or the production of fungitoxic derivatives within the plant². There is, however, evidence to suggest that WL 28325 acts by sensitising the host such that subsequent inoculation with *P. oryzae* results in a resistant reaction. Both the natural and the WL 28325-induced resistance of rice involve a hypersensitive type of browning reaction (as is commonly found in incompatible plant–fungus interactions) presumed to be a localised accumulation of melanoid pigments in the plant cells resulting from the peroxidase-mediated oxidation of phenols. This response is accentuated and accelerated by previous treatment of the plants through the roots with WL 28325 (ref. 3).

Microscopic observations of the infection process on

Table 2 Effect of inoculum and fertiliser phosphorus on mycorrhizal infection, foliar phosphorus and tuber yield

		Control seedbed (fallow soil)		Inoculum seedbed (cereal soil)	
Triple superphosphate (kg ha ⁻¹)		0	481	0	481
% Mycorrhizal infection*					
22 June		0.5 a	0.9 ac	8.3 b	4.0 bc
12 July		4.6 a	1.6 a	24.3 b	2.6 a
2 August		3.2 ac	1.2 a	24.5 b	5.9 c
% Phosphorus concentration in leaves					
12 July		0.16 a	0.19 a	0.17 a	0.17 a
Tuber yield (kg per plot)					
3 August		6.15 a	9.49 b	7.39 c	8.80 b

Means in any one row with the same letter do not differ significantly at *P* = 0.05.

*Means compared as arcsin transforms from percentage infection.

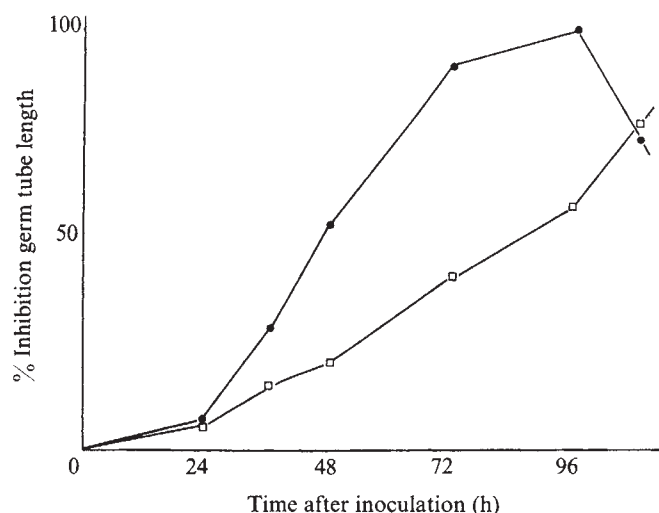


Fig. 1 Effect of WL 28325 treatment and *P. oryzae* infection on the inhibitory activity of drop diffusates against germ tube growth. ●, WL 28325-treated and infected; ○, untreated and infected. Each point is the mean of three replicate samples.

leaves showed that germination and appressorium formation by spores of *P. oryzae* were similar on WL 28325-treated and untreated plants. The internal development of the disease was examined in toluidine blue stained transverse sections of wounds on leaves of treated and untreated plants. In untreated leaves examined 48 h after inoculation, fungal hyphae were observed spreading throughout the vascular bundles of the inoculated wound. In the following 40 h the hyphae increased in number and spread into the tissue surrounding the wounded area. Hyphae were rarely observed in inoculated wounds of treated leaves, however, even 96 h after inoculation. This suggests that WL 28325-induced resistance of rice to *P. oryzae* might involve a more direct suppression of fungal growth rather than a physical restriction of the fungus to the inoculated area by the brown melanoid pigments as suggested previously³.

This possibility was substantiated when infection droplets were removed at intervals from the wounds on treated and untreated leaves and tested against germ tube growth of *P. oryzae*. When compared with spores germinated in distilled water, inhibition of germ tube growth was evident in diffusates collected from treated, infected leaves (Fig. 1). Diffusates collected from untreated, infected leaves were considerably less inhibitory while those collected from uninfected leaves, either treated or untreated, showed no antifungal activity. Repeated experiments confirmed that this antifungal activity induced by infection with *P. oryzae*, was enhanced by treatment of the plants with WL 28325 before inoculation.

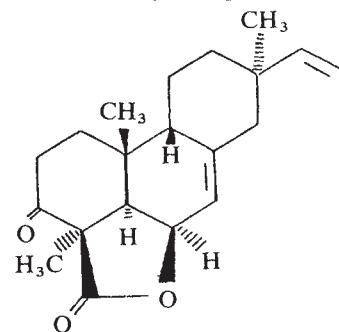
Similar results were obtained using ethanolic extracts of infected leaves rather than drop diffusates. The extracts were subjected to thin-layer chromatography (silica gel; solvent system, chloroform-ethanol (95:5v/v) followed by a direct bioassay for antifungal compounds using *Cladosporium cucumerinum*⁴. This revealed two major antifungal zones in chromatograms of extracts of infected leaves. Chromatograms of extracts of uninfected leaves showed no such inhibitory areas. Similar antifungal activity could be induced by ultraviolet irradiation of either leaves or dark-grown coleoptiles of rice. In these conditions antifungal activity was not influenced by treatment of the plants with WL 28325. Elution of the antifungal zones from the chromatograms showed that the areas of the chromatograms which inhibited *C. cucumerinum* also inhibited *P. oryzae*.

Fungal infection thus induces the production of antifungal compounds which may be defined as phytoalexins—antimicrobial compounds formed by plants in response to

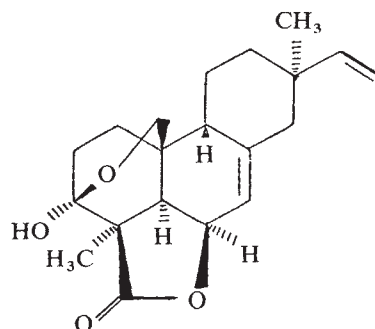
injury, physiological stimuli or the presence of infectious agents. As the phytoalexins are induced in untreated rice plants following ultraviolet irradiation they are clearly of host and not fungal origin and they are not antifungal derivatives of WL 28325.

The two major phytoalexins were isolated in small quantities as chromatographically homogeneous amorphous solids from ultraviolet-irradiated dark-grown rice coleoptiles by column chromatography on Sephadex LH-20 and preparative thin-layer chromatography on silica gel. Electron impact (EI) and field desorption (FD) mass spectrometry of the less polar compound showed a molecular ion at m/e 314. The more polar compound showed no clearly recognisable molecular ion by EI mass spectrometry but by FD mass spectrometry a presumed molecular ion at m/e 330 together with a strong $M+1$ ion was obtained. Detailed analysis of the 270-MHz proton magnetic resonance (PMR) spectra indicated their probable identity with momilactones A (I) and B (II). These two 9- β -pimaradiene diterpenes have been isolated as plant growth inhibitors from rice husks^{5,6}. Comparisons by combined gas-liquid chromatography (GLC)-mass and PMR spectroscopy of the phytoalexins isolated from rice with authentic samples subsequently confirmed their identity with the momilactones A and B. Optical rotation measurements showed that they also have identical stereochemistry. Yields of the momilactones A and B isolated from ultraviolet-irradiated coleoptiles were 3.8 and 0.9 mg per kg fresh weight respectively while estimated recoveries were 70 and 50% of the momilactone content of crude extracts. Combined GLC-mass spectroscopy confirmed the presence of the momilactones A and B in extracts of infected leaves and these two compounds account for all the antifungal activity of the crude extracts. Concentrations giving 50% inhibition of germ tube growth of *P. oryzae* for the momilactones A and B were 5 and 1 $\mu\text{g ml}^{-1}$ respectively.

We believe that this report is the first in which phytoalexins have been characterised from a member of the Gramineae, although there have been previous indications that phytoalexins may be produced by rice⁷ and



(I) Momilactone A



(II) Momilactone B

barley⁸. The diterpene nature of the momilactones is a significant departure from all previously described phytoalexins, these being mostly phenols or sequiterpenes⁹. A diterpene hydrocarbon from *Ricinus communis*, casbene, may have some of the properties of a phytoalexin although its production in response to infection was only reported for cell-free extracts¹⁰.

While certain fungicides are themselves capable of eliciting phytoalexin production¹¹, the activity of WL 28325 is unique in that the compound itself does not stimulate phytoalexin production but rather it increases the capacity of rice to synthesise phytoalexins in response to infection—this may be the basis for its disease-protectant properties. It is hoped that future studies on the effects of WL 28325 on the host-parasite relationship in the rice blast disease will not only provide further insight into the processes of natural resistance of rice to disease but will also indicate ways in which host defence mechanisms can be manipulated to control plant disease.

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Stimulation of egg-laying by nerve extracts in slugs

LITTLE is known about the mechanism of egg-laying in mollusca, although their reproduction is under endocrine control. In the freshwater snail *Lymnaea stagnalis*, cauterisation of the neurosecretory caudodorsal cells of the cerebral ganglia causes cessation of oviposition. Subsequent injection of a homogenate of cerebral commissures induces oviposition in all snails with caudodorsal cells. This suggests that the caudodorsal cells produce a hormone, which

stimulates oviposition¹. Kupfermann² induced ovulation and oviposition in *Aplysia californica* by injection of a homogenate of neurosecretory cells, the so-called bag cells, from the abdominal ganglion of this species. Although the morphology of the reproductive system has been studied in various terrestrial slugs^{3–7}, there are conflicting reports about the control of reproduction. I report here that homogenates of the central ganglia and tentacles, which are the sites of neurosecretory cells^{8,9}, stimulate egg-laying in the slugs, *Deroceras reticulatus* (= *Agriolimax reticulatus*) and *Limax flavus*.

Egg-laying in *D. reticulatus* occurs throughout the year, reaching peaks in April and October while in *L. flavus* the breeding season is May to November. I used slugs during their breeding season. Brains and tentacles were extirpated and homogenised in physiological saline. Then they were injected into the body cavity at a dose of four or five homogenised organs per slug. The rate of fertilisation, as represented by the hatching rate, of oviposited eggs was also examined. After 7 d oviposited eggs were counted.

The results are shown in Table 1. Brain hormone (homogenate of central ganglia) stimulated egg-laying and tentacular hormone (homogenate of tentacles) inhibited egg-laying in both species. To investigate the nature of the active principles in the homogenates, the effects of boiling and dialysis of each sample were studied. Table 1 shows that the results were similar to those for crude extracts—the treated homogenates of ganglia and tentacles still stimulated or inhibited egg-laying. The rate of fertilisation in the group given tentacular hormone was lower than that in the group given brain hormone or in the control groups. Table 2 shows the results of similar experiments on slugs with tentacles extirpated. Removal of tentacles induced a large increase in the number of eggs laid and an increase in the rate of fertilisation in both species. Results of hormone treatment were similar to those observed with intact slugs of both species. Accelerated oviposition and a high rate of fertilisation induced by removal of tentacles was inhibited by injection of tentacular hormone. The injection of brain hormone into slugs without tentacles induced still greater egg-laying and fertilisation in both species. Both hormones remained active in spite of boiling or dialysis. The minimum time between injection and the onset of egg-laying was 6 h. But the effects of the hormones continued for about 7 d. Egg-laying usually commenced 12 h after treatment. In *Lymnaea stagnalis*¹, the mean response latency was 131 min, *Aplysia californica*², it was 72 min but there was a seasonal fluctuation in amount of active principle and/or the ability to respond¹⁴. In *Lymnaea stagnalis*, injection of cerebral commissures into intact snails induced oviposition in most but not all recipients. In my experiments, some individual recipient slugs did not respond to the injection of brain extract.

These results suggest that brain hormone stimulates egg-laying directly, while tentacular hormone inhibits the brain

Table 1 Effects of the injection of brain and tentacular homogenates on oviposition in the slug, *Deroceras reticulatus* (D) and *Limax flavus* (L)

Materials injected	No. of slugs used		No. of eggs oviposited (rate of fertilisation)		Average no. of eggs per slug	
	D	L	D	L	D	L
Brain homogenates						
Crude	20	20	510 (72%)	420 (76%)	25.5	21.0
Heated	14	14	329	259	23.5	18.5
Dialysed	14	14	308	266	22.0	19.0
Tentacular homogenates						
Crude	20	20	210 (69%)	110 (74%)	10.5	5.5
Heated	14	14	77	105	5.5	7.5
Dialysed	14	14	63	112	4.5	8.0
Control (Physiological saline)	14	14	224 (85%)	189 (87%)	16.0	13.5

Table 2 Effects of the injection of brain and tentacular homogenates on oviposition in the slug, *Deroceras reticulatus* (D) and *Limax flavus* (L) without tentacles

Materials injected	No. of slugs used		No. of eggs oviposited (rate of fertilisation)		Average no. of eggs per slug	
	D	L	D	L	D	L
Brain homogenates						
Crude	20	20	640 (94%)	461 (93%)	32.0	23.0
Heated	14	14	406	280	29.0	20.0
Dialysed	14	14	427	273	30.5	19.5
Tentacular homogenates						
Crude	20	20	140 (63%)	120 (71%)	7.0	6.0
Heated	14	14	119	70	8.5	5.0
Dialysed	14	14	105	98	7.5	7.0
Control						
Tentacles cut off	14	14	350 (88%)	259 (81%)	25.0	18.5
Intact	12	12	144 (82%)	108 (78%)	12.0	9.0

hormone. The role of these hormones in fertilisation remains unclear.

Tentacles have been shown to be neuroendocrine organ⁸⁻¹³. In the tentacle, the tentacular ganglion is located close to the eye. It is connected with the procerebrum by the tentacular nerve. It seems likely that the tentacular hormone inhibits the activity of the neurosecretory cells of the central ganglia and as a result, egg-laying is inhibited. This resembles the hypothalamo-hypophysis-gonadal system in vertebrates. As the eye is located near the neurosecretory cells of the optic tentacle, environmental stimuli such as light and day-length, may influence the release of tentacular hormone.

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Differential pigmentation in the sexual phase of *Coelomomyces*

THE fungal mosquito parasite, *Coelomomyces dodgei* (Chytridiomycetes: Blastocladales), produces gametophytes and gametangia of two different colours, light amber and bright orange, in the intermediate copepod host, *Cyclops vernalis*. Each gametangium produces gametes of a corresponding colour. Experimental evidence is presented here which indicates that these differentially pigmented gametes are of opposite mating types, the light amber being female and the bright orange being male, and that zygotes capable of infecting mosquitoes, are produced by their fusion. This differential pigmentation provides a valuable marker for identifying gametes of opposite mating type for experimental purposes, and additionally provides further evidence for a phylogenetic relationship between *Coelomomyces* and certain other genera of the order Blastocladales.

Since the 1930s, fungi of the genus *Coelomomyces* have

been considered potential biological control agents for mosquitoes because of their well documented ability to produce epizootics in larval populations¹⁻³. This potential has not been realised or even accurately evaluated because of the difficulties of establishing laboratory cultures for detailed investigations. The primary reason for these difficulties became apparent during the past few years with the discovery that *C. psorophorae*^{4,5} and *C. punctatus*^{6,7}, parasites respectively of the mosquitoes *Culiseta inornata* and *Anopheles quadrimaculatus*, have alternate stages which develop in an intermediate host, the copepod, *Cyclops vernalis*. This finding led to the development of routine reproducible methods for the establishment of *in vivo* laboratory cultures of *C. psorophorae* and *C. punctatus* and more recently has resulted in the establishment of an *in vivo* culture of *C. dodgei*⁸ using *A. quadrimaculatus* and *Cy. vernalis* as alternating hosts.

Two of the above studies^{5,8} have also provided substantial evidence that these three species of *Coelomomyces* have an Euallomycetes type of life cycle with sporophyte and gametophyte generations alternating between the mosquito and copepod host, respectively. In essence, the evidence suggests the following general life cycle, although many details remain to be resolved, including precisely when meiosis occurs. The sporophyte in the mosquito larva produces meiosporangia which release meiospores after death of the host. The meiospores, in turn, infect copepods and develop into gametophytic thalli which subsequently become gametangia and produce gametes. Gametes fuse forming zygotes which infect mosquitoes and produce a sporophyte, thereby completing the life cycle. This cycle was first elucidated by Whisler *et al.*⁵ working with *C. psorophorae* and later by Federici and Chapman⁸ studying *C. dodgei*. The latter authors also observed gametophytes and gametes of two different colour types, light amber and bright orange, which apparently do not occur in *C. psorophorae*, and suggested that this differential pigmentation corresponded with opposite mating types. The present report provides experimental evidence for this hypothesis.

Copepods of *Cy. vernalis* were infected with *C. dodgei* using methods similar to those of Federici and Chapman⁸. Several hundred copepods were combined with about 5,000 dehiscing meiosporangia in 1 l of deionised water to which approximately 50 mg each of brewer's yeast and egg yolk had been added 48 h previously. Ten cultures were prepared in this manner and pooled 10 d later. The copepods were removed from the medium with a net every day from the 13th to the 18th day after exposure to the dehiscing meiosporangia. Patently infected adult copepods, which varied in colour from light amber to bright orange, were separated from the other copepods and the latter were returned to the medium. Infected copepods were examined individually with a phase microscope to confirm the colour

of the gametangium(a) in the haemocoel. The intensity of the colour, that is, the amount of pigmentation, frequently varied, especially in orange plants, from one copepod to another, but generally the infected copepods could be separated into three different types; those containing (1) only bright orange gametangia; (2) only light amber gametangia; and (3) both types. After separation into types, each copepod was placed in a 35×10 mm Petri dish in 2 ml filter-sterilised filtrate from the original copepod rearing medium. At least 20 copepods of each colour type were so prepared for each experiment. In most cases, the gametangia released the gametes, which were equal in size in both colour types, within 2 h. Shortly after gamete release, the copepod died and ruptured releasing gametes into the medium. The media containing gametes were then pooled in various combinations of type and amount and tested for mosquito infectivity. Unpooled media were also tested for infectivity. This was done by combining the media containing gametes with 20 recently hatched first-instar larvae of *A. quadrimaculatus* in a 60-mm Petri dish for 24 h. The larvae were then transferred to 1 l of rearing medium and examined periodically for infection. Additionally, drops of the pooled and unpooled media were examined periodically with a phase microscope for the presence of biflagellate planonts.

The results are shown in Table 1. No mosquito infection resulted in any test where larvae were exposed to gametes of a single colour type regardless of the number of gametes used. Some infection resulted in all tests where larvae were exposed to mixtures of light amber and bright orange gametes even in cases where both colour types were from the same copepod. Interestingly, the highest infection rates per copepod were obtained in tests where gametes from copepods containing both colour types were pooled, indicating greater numbers of zygotes may be formed if gametes of opposite mating type are produced in the same copepod.

Microscopic examinations were less definitive. In pools of gametes of a single colour type, no fusion was observed on pooling although occasionally bi- and multiflagellate planonts were observed. Although biflagellate zygotes were common in the mixtures of different coloured gametes, the actual process of fusion was observed only infrequently.

These results provide strong evidence for an *Euellomyces* type of life cycle for *C. dodgei* with the differentially pigmented sexual structures, light amber and bright orange,

being of opposite mating type. A similar type of sexual pigmentation is well known in several other *Blastocladias* with an *Euellomyces* type of life cycle. Where it occurs in the genus *Allomyces*^{9,10}, both female (unpigmented) and male (orange) gametangia occur on the same gametophytic plant, and the resultant female gametes are larger than the males. The case of *Blastocladiella variabilis*, however, more closely resembles the situation in *C. dodgei*. In *B. variabilis* Harder and Sörgel^{11,12} found that unpigmented and orange gametangia developed on separate plants and furthermore, that the resultant gametes, which were of opposite mating type, were equal in size.

The above reports provide a precedent for designating the light amber reproductive structures of *C. dodgei* as female and the bright orange as male. In summary then, *C. dodgei* seems to have an *Euellomyces* type of life cycle with sporophyte and gametophyte generations alternating between a definitive larval mosquito host and an intermediate copepod host, respectively. The meiosporangia which develop on the sporophyte release meiospores after mosquito death. The meiospores infect a copepod and each produces a separate gametophytic thallus which at maturity is converted to one or several gametangia, but releases only a single type of gamete. Female gametangia are light amber and release unpigmented gametes. Male gametangia are bright orange and release orange gametes. One or both sexes can develop in a single copepod depending on whether the infecting meiospore(s) form male or female thalli. Uniflagellate male and female gametes fuse in appropriate conditions forming a biflagellate zygote which completes the sexual cycle by infecting a mosquito larva and producing another sporophyte.

Differentially pigmented mycelia have also been observed in *Cy. vernalis* experimentally infected with *C. punctatus* and it is likely, therefore, that this species has a life cycle similar to that of *C. dodgei*.

The occurrence of differential sexual pigmentation in *Coelomomyces* should enhance efforts to evaluate the mosquito control potential of this fungal group. The orange pigmentation or lack of it can serve as a sexual marker for genetic studies directed towards selecting highly mosquito-infective strains and for investigations on the biosystematics of species of *Coelomomyces* in which this pigmentation occurs.

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Table 1 Infectivity of orange pigmented and unpigmented gametes of *C. dodgei*, alone and in combinations, for larvae of *A. quadrimaculatus*

Total no. of copepods	Colour type	No. of infected larvae out of 20 exposed in each test			Average % infection for the three tests
		Test no. 1	Test no. 2	Test no. 3	
1	Amber only	0	0	0	0
2	Amber only	0	0	0	0
4	Amber only	0	0	0	0
6	Amber only	0	0	0	0
1	Orange only	0	0	0	0
2	Orange only	0	0	0	0
4	Orange only	0	0	0	0
6	Orange only	0	0	0	0
2	1 Amber only + 1 orange only	7	1	4	20
4	2 Amber only + 2 orange only	8	1	6	25
6	3 Amber only + 3 orange only	9	4	7	33.3
1	Both amber and orange	4	1	2	11.6
2	Both amber and orange	4	1	3	13.3
3	Both amber and orange	11	2	5	30
6	Both amber and orange	14	3	9	43.3

Genetic variation in multigene families

THE possibility that some important quantitative characters of higher organisms may be subject to multigene families such as that of immunoglobulin variable parts¹, poses the problem of reformulation on some of the population genetics theory. The two remarkable characteristics of multigene families as shown by Hood and others^{1–3}—the contraction and expansion of the gene number in a family and the coincidental evolution—should now

be seriously taken into account in the theoretical investigation of the evolution of higher organisms. In addition, the unit of natural selection is the family itself rather than individual mutant genes. The model of unequal crossing-over provides one of the most satisfactory explanations for the coincidental evolution¹⁻⁴. In this model, the gene lineage gradually expands or contracts within a family mainly by intrachromosomal unequal crossovers. I have shown⁵ that the dynamics of a gene lineage within a chromosome under this model and some other models is analogous to that of a mutant gene in finite populations using the diffusion model of Kimura⁶. Here I present the equilibrium properties of genetic variation of multigene families under simplifying assumptions and show that natural selection may be quite effective in keeping a large amount of variation in the family such as that of variable parts of immunoglobulins.

Let us measure genetic variation of a gene family within a chromosome by the clonality⁴ (C) defined as the sum of squares of gene lineage frequencies within a family,

$$C = \sum_i x_i^2 \quad (1)$$

where x_i is the frequency of the i th gene lineage in the chromosome. Note that the clonality is equivalent to homozygosity in the analogous population genetics model. I also use my previous model⁵ of unequal crossing-over, in which duplication and deletion of one unit by unequal crossing-over occur alternately so that the total number of units, n , in a chromosome is kept constant. Two successive crossovers (duplication and deletion) are called one cycle of the process.

Now the mean change of C by one cycle of crossing over has been shown to be⁵

$$E\{\Delta C\} = 2(1-C)/n^2 \quad (2)$$

where E stands for taking the expectation. In other words, C increases by an amount of $2(1-C)/n^2$ through one cycle of crossover. On the other hand, it decreases by mutation and there is therefore a stable equilibrium between mutation and unequal crossing-over. Let us assume that all mutations are unique and not pre-existing and let ν be the mutation rate per gene unit per generation. Let us further assume that k is the effective number of cycles of unequal crossing-over per chromosome per generation. The changes in clonality per generation by mutation (ΔC_ν) and by crossover (ΔC_k) are then expressed as

$$\Delta C_\nu = -2\nu C \quad (3)$$

$$\Delta C_k = 2k(1-C)/n^2 \quad (4)$$

Note again the analogy of this formulation with that of genetic variability in finite populations by Kimura and Crow⁷. The equilibrium value of C becomes

$$\hat{C} = k/(k+n^2\nu) \quad (5)$$

If k is proportional to n

$$\hat{C} = a/(a+n\nu) \quad (6)$$

where $a = k/n$. So far I have considered a single chromosomal family. Actually, however there are many chromosomal families in the population and ordinary crossing over occurs between the chromosomes. This would decrease the above $\hat{C}$ value. It would be expected that if the population is very large and if the inter-chromosomal exchange is frequent, the average clonality would be greatly reduced. Here if one assumes that the rate of intra-chromosomal crossover is much larger than that of inter-chromosomal crossover as has been assumed by Smith⁸, the present formulation may still apply approximately. The more general treatment is in progress. I shall show later some results of the simulation studies.

Let us now incorporate natural selection between chromo-

somes into the model. Let s be the selective disadvantage at $C = 1$ such that the fitness of a family with clonality C ($\bar{W}$) is equal to

$$\bar{W} = 1 - sC \quad (7)$$

For simplicity, the additive effect of clonality is assumed. Then the mean fitness of the population is

$$\bar{\bar{W}} = 1 - s\bar{C} \quad (8)$$

and the change in fitness is proportional to the change in C , $\Delta\bar{W} = -s\Delta\bar{C}$. The fundamental theorem of natural selection by Fisher⁹ states that the rate of change of mean fitness of the population is equal to the genetic variance of fitness in the population at that time. In our case, the change of mean fitness per generation may be expressed as

$$\Delta\bar{\bar{W}} = s^2 V_c / \bar{\bar{W}} \quad (9)$$

where V_c is the variance of clonality among the chromosomes in a population, hence the change in mean clonality per generation due to selection becomes,

$$\Delta\bar{C}_s = -sV_c / \bar{\bar{W}} \quad (10)$$

In other words, the mean clonality decreases by an amount $sV_c / \bar{\bar{W}}$ each generation by this type of selection.

This variance in clonality among the chromosomes (V_c) is the crucial factor which determines the effectiveness of natural selection and represents genetic variability in the sense of population genetics. At the moment, however it seems to be difficult to evaluate V_c . In the population genetics analogy, each chromosome corresponds to subpopulations of lineages, and inter-chromosomal crossing over to migration of mutant genes. Under the simplifying assumptions that the inter-chromosomal crossover is negligibly rare compared to the intra-chromosomal crossing over and that the population size is large without selection, V_c is expected to be roughly equal to Stewart's formula¹⁰ for the variance of heterozygosity in population genetics

$$V_c \approx \frac{2\theta}{(1+\theta)^2(2+\theta)(3+\theta)} \quad (11)$$

in which $\theta = n^2\nu/k$. Such an assumption may not usually be met although my preliminary simulation studies indicate that V_c is not much smaller in magnitude than the above formula suggests relative to the mean clonality even when the assumptions are not met. Thus, by comparing the formulas (3) and (10), it can be seen that natural selection may be more effective than mutation in reducing the average clonality or in increasing genetic variation in the chromosome, since the selection coefficient (s) is much larger than the mutation rate (ν).

I present here some of the results of my preliminary simulation studies. Since the details will be published elsewhere, only the parameters and the results are given. Two cases have been studied: (1) the population size (N) = 50 (haploid), $n = 10$, $\nu = 0.004$ and $k = 1$, (2) $N = 25$, $n = 5$, $\nu = 0.002$ and $k = 1$. Each case was run with and without selection ($s = 0$ or 0.5), and with and without inter-chromosomal crossover (total of 0 or 25 recombinations per generation in case 1 and 0 or 12 recombinations in case 2). Table 1 shows the results (average values of 101–350th generations). It can be seen that the present formulation holds without inter-chromosomal crossover. The average clonality decreases considerably with inter-chromosomal crossover though selection works whether there is inter-chromosomal crossover or not.

The present model is overly simple in another respect since it assumes that selection is solely dependent on the clonality and also that the number of gene units in a family is fixed. Actually,

Table 1 Average and variance of clonality observed in simulation studies

Population size (N)	No. of units (n)	Selection coefficient (s)	Total no. of inter-chromosomal crossovers per generation	Observed mean clonality	Observed variance of clonality
50	10	0	0	0.754	0.048
				(0.714)*	(0.050)†
		0.5	25	0.444	0.021
		0	0	0.433	0.016
25	5	0.5	25	0.295	0.009
		0	0	0.949	0.014
				(0.952)*	(0.015)†
		0.5	12	0.781	0.028
			0	0.710	0.034
			12	0.604	0.029

*Calculated by the formula (5).

†Calculated by the formula (11).

the selection is not only a function of clonality but also of the combinations of mutant genes, and also the number of gene units should be a random variable and subject to natural selection. In addition, the additive selection based on clonality is too simple. In principle, the fitness of a heterozygote could be assigned by measuring the clonality in the two chromosomes, which may differ from the additive effect. The more realistic approach awaits future analyses taking these assumptions into account. The important differences of the present approach from the traditional evolutionary theory are that the mutant genes can spread via coincidental evolution and that the gene family rather than individual mutant is considered as the unit of selection. Furthermore, in the present model, the genetic variation for natural selection to operate on (V_c) is generated by mutation and crossing-over, yet the total genetic variation including within-chromosome variance ($1-C$) may be kept mainly by selection. In fact, the antibody combining sites of immunoglobulins are hypervariable (see ref. 11 for review), suggesting that the kind of selection studied here is important. If multigene families are fairly common phenomena as speculated by Hood *et al.*¹, some similar type of selection may be operating in guiding some of the important quantitative genetic variations for the adaptation and evolution of higher organisms.

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Loss of ability to kill *Fasciola hepatica* in sensitised rats

RATS infected orally with metacercariae of the liver fluke *Fasciola hepatica* develop a partial resistance to oral challenge. This resistance occurs when the sensitising flukes have reached and are living in the bile ducts; resistance also develops if the sensitising infection is terminated by anthelmintics whilst the flukes are migrating through the liver parenchyma^{1–3}. Living adult flukes can be successfully

transplanted into the peritoneal cavity of rats^{4,5}, but if the rats have been infected with *F. hepatica* metacercariae approximately 10 weeks previously, the flukes transferred into the peritoneal cavity will be killed^{2,6}. We report here that although a marked resistance to intraperitoneal challenge with adult flukes develops as early as 2 weeks after oral infection with normal metacercariae, rats which have been infected 12 months previously, and which still have living adult flukes present in their bile ducts have lost this immunity. If rats with long-standing fluke infections in their bile ducts are reinfected with metacercariae, however, the ability of the rats to kill the challenge flukes is restored.

Adult flukes, obtained aseptically from the bile ducts of donor goats, were kept individually until transfer at 37 °C in medium 199 containing 10% foetal calf serum and antibiotics⁵. Female PVG rats (Piebald Virol Glaxo) were each infected with 20 metacercariae. The faeces of rats with long-standing infections were examined for fluke eggs as previous work⁷ had shown that many rats eliminate a primary infection after 7–8 months. Control and sensitised rats received three living adult flukes intraperitoneally. The rats were killed 7 d later and flukes were recovered and examined.

The results given in Table 1 show the effect of the duration of a primary infection on subsequent challenge. Rats sensitised for 1 week only did not kill the adult

Table 1 The effect of duration of infection on subsequent challenge

Infection and duration	No. of rats	Total no. of flukes found at necropsy		
		Dead	Alive	% Dead
None (Control 1)	6	0	18	0
None (Control 2)	6	2	16	11
1 week	5	1	14	6
2 weeks	5	13	2	86
3 weeks	6	13	5	72
4 weeks	6	17	1	98
6 weeks	6	15	3	83
12 months	6 (–ve)*	0	18	0
12 months	6 (+ve)*	3	15	17
None (old rats) control	5	0	15	0

Each rat was challenged with three living adult flukes placed in the body cavity. These are the flukes recovered at post mortem examination and referred to in the Table. Two control groups (1 and 2) are shown as the Table summarises two experiments. The group of old control rats was included to show that surgically transferred flukes behave normally in old rats.

* (+ve) or (–ve) indicates the presence or absence of flukes in bile ducts from primary infection.

Table 2 Effect of reinfection on subsequent challenge

Age of primary infection	No. of rats	Flukes in bile ducts	Postmortem examination		% Dead
			No. of challenge flukes Dead	Alive	
12 months	7(+ve)	present	17	4	84
12 months	7(-ve)	absent	19	2	94
None (control) (old rats)	6	—	18	0	100

The first group of rats (+ve) showed fluke eggs individually in their faeces whereas the second group were negative. These two groups of rats together with uninfected controls were infected with 20 metacercariae orally and all rats challenged 3 weeks later with three adult flukes. At post mortem examination the flukes found in the bile ducts were from the primary infection. All rats showed fluke tracts on their livers.

challenge flukes placed in the body cavity, but by two weeks after sensitisation this ability to kill the challenge had fully developed and was also seen to be operative at 3, 4, and 6 weeks after sensitisation. In contrast, rats which had been infected 12 months previously had lost the ability to kill challenge flukes in spite of the continued presence of adult flukes of the primary infection in the bile ducts.

Table 2 demonstrates the re-infection with metacercariae restores the ability to kill flukes in rats infected 12 months previously, irrespective of whether the primary infection had been eliminated or not. In those rats in which adult flukes of the primary infection were still living in the bile ducts (+ve rats in Table 2), the new sensitising secondary infection of 3 weeks duration was also alive and actively migrating in the liver parenchyma, whereas the challenge flukes were killed in the body cavity.

The ability of rats to kill the adult challenge flukes was apparently dependent on a recent migration of immature flukes in the liver parenchyma. It has been suggested that the duration of liver migration (10–11 d) in mice is responsible for the stimulation of acquired immunity⁸. Rats in which flukes have reached the bile ducts progressively lose their ability to kill challenge flukes. Perhaps the adult flukes living in the bile duct either do not produce the right antigenic stimulus, or do not present it in the right situation for processing. Alternatively, the migratory phase in the liver may produce an inflammatory response of short duration acting in the body cavity. Preliminary histological observations on the cysts surrounding challenge flukes in sensitised animals showed an intense infiltration of eosinophils into the walls and lumen of the cysts.

Although it is dangerous to extrapolate from a small animal model system, it is possible that the situation seen in the field whereby ruminants such as sheep or goats with long standing fluke infections are susceptible to re-infection is similar to the phenomenon reported here.

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Microcalorimetry of isolated mammalian cells

USING microcalorimetry^{1,2}, we have measured the heat evolved by isolated cells of brown adipose tissue (BAT). This tissue generates heat in newborn and cold-adapted mammals and in hibernators during arousal from hibernation and during cold stress^{3–5}. The method can be applied generally in work with, for example, mitochondria and isolated cells and has, especially in studies of thermogenesis,

the advantage of measuring the gross calorific output of all processes in the cell. Our results, compared with others obtained by routine methods, show that it is an oversimplification to consider BAT as the major source of heat during arousal.

Noradrenaline (NA) is the physiological stimulator of thermogenesis in BAT, and the brown adipocytes responded calorigenically (Fig. 1) to stimulation with NA in accordance with the patterns known from polarographic studies. NA-induced oxygen consumption proceeds faster and for a longer time if the buffer is bubbled with 5% CO₂ in air, rather than with air⁶, and this is reflected in our measurements of calorific output (Fig. 1). Because oleate stimulates oxygen consumption in adipocytes, possibly by mimicking the action of the hormone-sensitive lipase⁷, we have investigated the calorific effect of 35 μ M oleate. The response of the cells paralleled those obtained by polarographic studies (not shown).

Table 1 Ratios of heat evolution to oxygen consumption

Theoretical	
NADH oxidation to water	258
Succinate oxidation to water and fumarate	151
Palmitate, stearate or oleate combustion to water and CO ₂	218
Acetate combustion to water and CO ₂	219
Palmitate oxidation to acetate and CO ₂	219
Experimental	
1 μ M NE-stimulated cells	243

The ratios are expressed as kJ per mol of oxygen. The theoretical values are calculated from¹⁴. The experimental value is calculated from the steady state 5 min after stimulation. (Ivlevs oxy-calorific coefficient¹⁵ amounts to 226 kJ per mol of oxygen.)

The heat evolved by maximal NA-stimulation at 37 °C was 820 μ W per 10⁶ cells, and heat evolution continued for at least 50 min. This is five times greater (using our estimate of 10⁶ cells per g wet weight) than reported in the only other calorimetric study, made with tissue slices⁹. Based on these figures the 2 g of BAT found in a hamster weighing 100 g (with the same heat capacity as water) would be able to warm the whole hamster less than 1 °C per h (neglecting in the calculation heat loss to the surroundings). This should be contrasted to the fact that hibernating hamsters arouse, even in a cold environment, in 2 h, that is, they increase their body temperature 15 °C per h, and even though some shivering takes place in the last phase, BAT itself is clearly not able to deliver a substantial amount of the heat needed. Furthermore, our experiments were carried out at 37 °C. The heating capacity of BAT in a hibernating animal with lowered body temperature would be even less. To illustrate this we compared the NA-evoked oxygen consumption in cells at 37 °C with that at lower temperatures and found it to be decreased to 67% at 27 °C, to be less than 10% at 15 °C and to be immeasurable at 7 °C, the body temperature of hibernating hamsters.

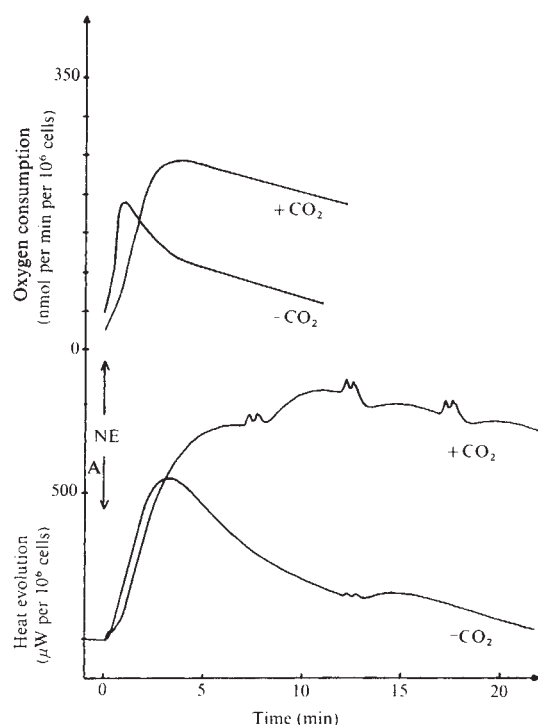


Fig. 1 Comparison between polarographic and calorimetric responses of brown adipocytes to stimulation with NA. Brown adipocytes were prepared as described before⁶ by collagenase treatment of pooled BAT from warm-adapted hamster. (In contrast to non-hibernators such as the rat, hamsters and other hibernators retain thermogenic BAT even in a warm environment.) Oxygen consumption was measured polarographically⁷ at 37 °C with a Clark oxygen electrode. Heat evolution was measured in a LKB 2107 batch microcalorimeter. The sample vessel consisted of a large compartment (4 ml) containing the cell suspension and a small compartment (1 ml) containing NA. The reference vessel contained cell suspension in the large compartment but only buffer in the other. The temperature of the thermostat was 37 °C. After equilibration, the contents of the vessels were mixed by rotation, and the difference in heat output between stimulated and unstimulated (basal respiring) cells was monitored on a potentiometric recorder. The instrument was calibrated by applying a known current through a known internal resistance. The response time of the calorimeter was in the order of tens of seconds. Control experiments showed that an unlimited supply of oxygen to the cells was ensured if the vessels were rotated every fifth minute, enabling the oxygen in the vessels to dissolve in the buffer. This was done in all experiments, giving rise to the distortions on the figures. Heat of dilution of agents has been subtracted from the curves where necessary. All cell suspensions were at 100,000 per ml in modified⁶ Krebs-Ringer phosphate buffer (unless otherwise stated the buffer was bubbled before the experiments with 5% CO₂ in air). NA was added to a final concentration of 1 μM for maximal stimulation.

Heat production is generally assumed to be proportional to oxygen consumption, but the heat produced per oxygen molecule used varies in different processes, for example, glycolysis and oxygenase reactions. Calculations¹⁰ of mitochondrial bioenergetics demonstrate a very high efficiency for formation of ATP, up to 96% energy conservation. If the ATP formed is not immediately hydrolysed in a process where the energy is not conserved, the ratio of heat: oxygen will be low. Thus estimation of heat evolution from oxygen consumption could be misleading, and we have therefore measured directly the caloric output and compared it with oxygen consumption.

Table 1 shows the calculated and measured ratios of heat evolution to oxygen consumption. The values are consistent with expectation. If CO₂ was omitted the ratio was not affected. This method does not enable us to distinguish between partial and total combustion of fatty acids^{11,12}. We find, as did Peakin¹³, that respirometry is a satisfactory method to estimate heat production.

To justify the use of isolated cells or mitochondria as model systems for the elucidation of the functions of BAT *in vivo*, we tabulated data from various laboratories as well as from different animals (Table 2). As conversion factors we used 10⁸ cells and 130 mg of mitochondrial protein per g of BAT (wet weight), obtained from morphometric studies, cytochrome oxidase measurements and protein determinations (personal communication from T. Barnard and B. Pettersson). We found a good correlation between systems. Earlier failures^{16,17} to obtain a satisfying oxidative capacity of BAT mitochondria have been due to the use of an 'unphysiological' substrate for mitochondria (2-oxoglutarate instead of fatty acids). Only in the case of glucose uptake did we find a discordance, and we offer two explanations for this: (1) glucose permease is destroyed during cell preparation by the crude collagenase, known to have protease activity, and (2) the value from the whole animal is calculated as arteriovenous difference. The endothelium, the nerve endings and the mast cells—perhaps 10% of the tissue altogether—may utilise most of the glucose, the adipocytes using mainly fatty acids for their basal metabolism.

In newborn rabbits²⁸ and lambs²⁹ BAT accounts for most of the increase in oxygen consumption during cold-stress or infusion of NA. In cold-adapted rats it is suggested to account for 8% of the increased oxygen consumption³⁰. No direct measurements have been made on hibernators. Jansky³¹ has estimated the importance of BAT during arousal from hibernation (in bats). He measured the oxidative capacity of cytochrome aa₃ and concluded that BAT could consume 25% of the total oxygen used during arousal. This is a maximum estimate—cytochrome aa₃ is presumably not the rate-limiting enzyme in fatty acid

Table 2 Comparison between quantitative results from investigations on different organisation levels

	Heat production experimental (W)	Heat production calculated from oxygen consumption (W)	Oxygen consumption (μmol min ⁻¹)	Fatty acid release (μmol min ⁻¹)	Fatty acid uptake (nmol min ⁻¹)	Glucose uptake (nmol min ⁻¹)
Mitochondria		0.6	182 (Hamster) ^{18*} 174 (Rat) ¹⁹ 170 (Lamb) ²⁰ 226 (Guinea pig) ²¹			
Adipocytes	0.2	0.3	100 (Hamster) ⁸	8 (Hamster) ²³	400 (Hamster) ²⁵	12 (Rat) ²⁷ 120 (Hamster) ²⁵
Whole animal	See text		100 (Rabbit) ²²	4 (Rabbit) ²⁴	240 (Rabbit) ²⁶ 770 (Rabbit) ²⁴	3,000 (Rabbit) ²⁴

All values are related to 2 g of BAT as found in a newborn rabbit or an adult hamster (both 100 g). As mitochondria are traditionally examined at 23 °C, the values here have been corrected to 37 °C by a factor 2.8, obtained from experiments with hamster mitochondria (our unpublished observation and ref. 11).

*All uncoupled palmitoyl carnitine oxidation in the presence of malate.

combustion.

Our calorimetric measurements indicate that BAT has a low thermogenic capacity when compared with the heat needed to arouse a torpid hamster. Because of the general accordance between data obtained from studies *in situ* and in isolated cells, we believe that the cells elicited their full thermogenic capacity, and the opinion that BAT has thermogenesis as its primary function requires modification. Several suggestions have been made, for example, that the heat produced primarily warms the central nervous system¹ or that BAT also has an endocrine function³². During cold-stress the fat depots in BAT are emptied rapidly in both warm-adapted²⁵ and cold-adapted hamsters (our unpublished observations). As brown adipocytes are efficient in export of fatty acids^{23,24} which themselves have been shown to increase oxygen consumption in, for example, heart tissue³³, BAT may generate the main part of 'its' heat in other tissues where fatty acids liberated from BAT are combusted during cold stress. We have confirmed earlier studies³⁴ showing that in hamsters BAT does not lose any of its stored fat during arousal from hibernation. Because of these findings, and because of the relatively low calorific output reported here the direct role of BAT during arousal in the hamster must be questioned.

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Lymphocyte migration into cell-mediated immune lesions is inhibited by trypsin

THE lymph nodes and the white pulp of the spleen are major sites into which lymphocytes migrate from the blood and each organ is equally selective in that other blood cells are efficiently excluded^{1,2}. It might be expected that the same mechanism for discriminating between lymphocytes and other cells would have evolved in the spleen and lymph nodes, but the entry of lymphocytes into each of these organs can be distinguished in two ways. First, the histological appearance of the vascular endothelium across which lymphocytes migrate is dissimilar^{1,3} and second, the capacity of lymphocytes to migrate from the blood into lymph nodes is reduced by brief exposure *in vitro* to low concentrations of trypsin, but migration of the same population into the spleen is barely affected⁴. Lymphocytes are not irreversibly damaged by trypsin because they regain their capacity both *in vivo* and *in vitro* to localise in lymph nodes. The reduced numbers in lymph nodes is not a consequence of increased localisation of trypsinised lymphocytes elsewhere, as is testified by perfusing trypsinised lymphocytes through an isolated lymph node⁶ and also by measuring the numbers of trypsinised lymphocytes in the blood after intravenous injection^{6,7}. For several hours the concentration of treated cells in the blood was about 1.4 times that of control cells. This excess was attributed to the failure of trypsinised cells to enter lymph nodes⁶. We have extended these observations by comparing the capacity of trypsinised and control lymphocytes to migrate from the blood into several non-lymphoid tissues including sites of inflammation. Exposure of lymphocytes to trypsin not only exerts a differential effect on lymph node and spleen localisation, it also differentiates the mechanism by which lymphocytes migrate in small numbers into normal skin and other non-lymphoid tissues (trypsin resistant) from that responsible for the migration of increased numbers of lymphocytes into cell-mediated immune (CMI) lesions in the skin (trypsin sensitive).

To determine whether the differential effect of trypsin on lymph-node and spleen localisation is merely a quantitative effect, lymphocytes obtained by thoracic duct cannulation⁸ of AO rats were labelled *in vitro* with either ³H-uridine or ¹⁴C-uridine⁸. The ³H-labelled lymphocytes were exposed *in vitro* to graded doses of trypsin in phosphate-buffered saline (PBS), washed, combined with the ¹⁴C-labelled cells as a reference population and injected into syngeneic recipients. No reduction of spleen localisation was found with a concentration of trypsin 125 times greater than that needed to inhibit lymph-node localisation by 50% (Table 1). The standard concentration of trypsin previously used (0.02 mg ml⁻¹) (refs 4 and 6) reduced lymph-node localisation to 10% of untreated cells at 1 h after injection and increasing the dose by 5- or 25-fold had little additional effect on either the deficit in the lymph nodes or the excess in the blood. The distribution of trypsinised cells at 24 h resembled that of control cells and only at the highest concentrations was there evidence of loss of a minority of the cells. There thus seems to be a qualitative difference in the trypsin sensitivity of lymphocyte migration into the spleen and lymph nodes.

Lymphocytes also migrate from the blood into non-lymphoid tissues such as skin, albeit in much smaller numbers than into secondary lymphoid organs and the rate of migration is increased into sites of CMI (refs 9-12). We investigated whether migration into non-lymphoid tissue resembled migration into lymph nodes in being trypsin sensitive and whether trypsin treatment could distinguish between migration into normal and inflamed skin.

Lymphocytes were obtained by thoracic duct cannulation

Table 1 Effect of graded concentrations of trypsin on lymphocyte localisation in organs of recipients*

Ratios of ^3H (associated with trypsinised cells) to ^{14}C (control cells)†		Concentration of trypsin (mg ml ⁻¹)			
Time after injection	Organ	0.5	0.1	0.02	0.004
1 h	Lymph nodes	0.07	0.07	0.10	0.51
	Spleen	0.99	0.96	1.02	1.02
	Blood	1.47	1.46	1.32	1.17
	Lung	1.68	1.04	1.12	0.98
	Liver	1.35	1.22	1.01	0.86
	Gut	0.72	0.91	1.02	0.96
	Bone marrow	0.78	0.97	1.07	0.98
	Lymph nodes	0.72	0.76	0.89	0.89
	Spleen	1.18	1.06	1.05	0.92
	Blood	0.69	0.75	0.82	0.92
24 h	Lung	0.82	0.88	0.88	0.95
	Liver	1.15	0.91	1.07	1.04
	Gut	0.86	0.78	1.01	0.94
	Bone marrow	0.79	0.84	0.88	0.78

*Lymphocytes exposed to trypsin *in vitro* for 10 min.

†All ratios are standardised so that $^3\text{H}/^{14}\text{C}$ ratio in the injection sample = 1.00. A supplementary group of recipients received ^3H -labelled cells (untreated) and ^{14}C -labelled cells (untreated). As expected the $^3\text{H}/^{14}\text{C}$ ratio generally fell in the range of 0.90–1.10 indicating an even distribution. In the Table, a ratio of less than 0.80 indicates a significant deficit of trypsinised cells and a ratio of more than 1.20 a significant surplus.

of inbred AS or (PVG/c×DA) F_1 rats⁸. They were radioactively labelled *in vitro* by incubation with ^{51}Cr -sodium chromate at $10 \mu\text{Ci ml}^{-1}$. The cells were suspended in medium RPMI 1640 with 10% FCS at 37 °C for 1 h. After washing, half the suspension was treated with trypsin (Boehringer) at 0.02 mg ml^{-1} for 10 min. After further washing, trypsinised and control cells were injected intravenously into separate syngeneic recipients, which were killed at 0.5, 2 or 24 h after injection. A 2-ml sample of blood was taken and the entire vasculature was perfused through an aortic cannula with a large volume of PBS. The use of ^{51}Cr as a radioactive label for lymphocytes and the perfusion procedure have been found to be the most satisfactory method of measuring lymphocyte migration into non-lymphoid tissues¹³. The spleen and the six largest superficial cervical lymph nodes were removed and also samples taken of the tissues referred to in Table 2. The specimens were weighed before radioassay in a LKB γ -counter.

Two series of experiments were performed involving recipients with contact sensitivity lesions and adjuvant granulomata. Contact sensitivity was induced by painting an area of ventral abdominal skin with 0.1 ml of 5% DNCB in alcohol. Twelve days later a site on the back was painted

with a challenge dose of DNCB (0.1 ml of 0.5%). As control sites, primary application of two other contact sensitising agents, TNCB (0.1 ml of 1%) and oxazolone (0.1 ml of 0.5%), and croton oil (0.1 ml of 10%) were applied simultaneously to different areas of dorsal skin 24 h before the injection of labelled lymphocytes. In other recipients adjuvant granulomata had been produced by intradermal injection 21 d previously of 0.1 ml of Freund's complete adjuvant (Difco) emulsified with PBS.

The localisation of lymphocytes in normal skin was clearly not impaired by trypsin; radioactivity associated with trypsinised lymphocytes found in the skin soon after injection was equal to or slightly greater than radioactivity associated with untreated cells (Tables 2, 3) possibly reflecting the marginally higher numbers in the blood. It was confirmed that the migration of lymphocytes into the spleen and liver is unimpaired by trypsin treatment especially when compared with the blood level. Similarly, migration into muscle (Table 2), brain and ovary were almost equal for trypsinised and control cells suggesting that migration of lymphocytes into non-lymphoid tissues in general may be trypsin resistant. By contrast, migration into lymph nodes was reduced by trypsin to 1–2% of control values at 30 min or 2 h after injection (Table 2).

The most striking result was that trypsin treatment impaired the capacity of lymphocytes to migrate into sites of CMI in the skin (Tables 2 and 3). In the case of the DNCB lesion, the early localisation (0.5 h, 2 h) of untreated lymphocytes was increased to 1.5–2.5 times that of normal skin or sites painted with TNCB or oxazolone (Table 2). Although small, this increase was significant and occurred consistently in five other series of experiments on the migration of lymphocytes into DNCB lesions. The localisation of trypsinised cells was significantly less than that of control cells in DNCB lesions and was nearly the same as in normal skin. Thus, if it is acceptable to regard the values for normal skin as a baseline, the data indicate that very few if any trypsinised lymphocytes entered the skin as a result of DNCB challenge. The results followed the same pattern for the granuloma (Table 3) and were more emphatic: 2–3 times as many untreated lymphocytes were present in the lesions as were present in normal skin at 30 min and 2 h but equal numbers of trypsinised lymphocytes were present in the granuloma and in normal skin at each of the early intervals.

We conclude that mechanisms of selective lymphocyte migration can be placed into two categories. The first, which is trypsin resistant, operates in the spleen and non-lymphoid tissues and the second is trypsin sensitive and operates in lymph nodes and sites of CMI. Non-immune chronic inflammatory lesions have not been investigated.

It is possible that trypsin distinguishes two subsets of thoracic duct lymphocytes rather than two mechanisms of

Table 2 Effect of trypsin on ^{51}Cr -labelled lymphocyte localisation in contact sensitivity lesions
% injected activity per g tissue $\pm$ s.e.

	0.5 h <i>n</i> = 7		2 h <i>n</i> = 4		24 h <i>n</i> = 4	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
Normal skin	0.029 $\pm$ 0.004*	0.032 $\pm$ 0.004†	0.013 $\pm$ 0.003	0.013 $\pm$ 0.002	0.014 $\pm$ 0.001	0.011 $\pm$ 0.001
DNCB' (<i>n</i> = 16)	0.044 $\pm$ 0.003†	0.034 $\pm$ 0.002§	0.032 $\pm$ 0.011	0.020 $\pm$ 0.003	0.052 $\pm$ 0.015	0.041 $\pm$ 0.010
Croton oil	0.028 $\pm$ 0.005	0.027 $\pm$ 0.005	0.018 $\pm$ 0.004	0.018 $\pm$ 0.004	0.021 $\pm$ 0.003	0.021 $\pm$ 0.004
TNCB	0.020 $\pm$ 0.001	0.020 $\pm$ 0.003	0.012 $\pm$ 0.002	0.011 $\pm$ 0.001	0.012 $\pm$ 0.002	0.012 $\pm$ 0.002
Oxazolone	0.024 $\pm$ 0.005	0.023 $\pm$ 0.002	0.012 $\pm$ 0.002	0.014 $\pm$ 0.003	0.014 $\pm$ 0.002	0.013 $\pm$ 0.001
Blood leukocytes	0.41 $\pm$ 0.09	0.51 $\pm$ 0.08	0.07 $\pm$ 0.007	0.15 $\pm$ 0.03	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01
Lymph nodes	11.3 $\pm$ 3.11	0.11 $\pm$ 0.01	16.8 $\pm$ 4.4	0.35 $\pm$ 0.08	58.2 $\pm$ 8.7	51.6 $\pm$ 7.6
Spleen	82.1 $\pm$ 5.6	104.7 $\pm$ 7.9	94.6 $\pm$ 7.9	139.6 $\pm$ 8.2	50.8 $\pm$ 2.7	52.5 $\pm$ 2.1
Liver	2.1 $\pm$ 0.33	2.8 $\pm$ 0.45	1.2 $\pm$ 0.18	1.5 $\pm$ 0.07	1.2 $\pm$ 0.05	1.3 $\pm$ 0.05
Muscle	0.013 $\pm$ 0.002	0.019 $\pm$ 0.002	0.006 $\pm$ 0.001	0.008 $\pm$ 0.002	0.006 $\pm$ 0.001	0.004 $\pm$ 0.001
Bone marrow	3.5 $\pm$ 0.35	2.5 $\pm$ 0.33	3.7 $\pm$ 0.25	2.5 $\pm$ 0.05	0.88 $\pm$ 0.11	0.69 $\pm$ 0.12

*Against †, $P < 0.05$; † against §, $P < 0.02$; ‡ against §, not significant by the Mann-Whitney U test.

Table 3 Effect of trypsin on ^{51}Cr -labelled lymphocyte localisation in adjuvant granuloma
% injected activity per g $\pm$ s.e.

	0.5 h (n = 2)		2 h (n = 2)		24 h (n = 2)	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
Normal skin	0.027 $\pm$ 0.006	0.037 $\pm$ 0.001	0.021 $\pm$ 0.005	0.025 $\pm$ 0.002	0.020 $\pm$ 0.002	0.016 $\pm$ 0.002
Granuloma (n = 8)	0.069 $\pm$ 0.004*	0.038 $\pm$ 0.002†	0.039 $\pm$ 0.002‡	0.021 $\pm$ 0.001§	0.27 $\pm$ 0.02	0.10 $\pm$ 0.01
Blood leukocytes	0.42 $\pm$ 0.19	0.69 $\pm$ 0.08	0.10 $\pm$ 0.02	0.12 $\pm$ 0.003	0.21 $\pm$ 0.015	0.10 $\pm$ 0.034

Other tissues similar to Table 2.

*Against †, $P < 0.002$; ‡ against §, $P < 0.002$ by the Mann-Whitney U test.

selective migration which are properties of different endothelia. In particular, could the inhibitory effect of trypsin on migration into sites of CMI be exerted solely on large lymphocytes which are known to migrate especially well into sites of inflammation¹⁰⁻¹²? The present results do not completely exclude this but we feel it is likely that trypsin does impair small lymphocyte migration into CMI lesions for two reasons. First, small lymphocytes do migrate into CMI lesions in increased numbers as has been supported by cannulation of the lymphatic draining an adjuvant granuloma in sheep⁹ and selectively labelling small lymphocytes with ^3H -thymidine *in vivo* and detecting an increased localisation in DNCB lesions (G.H.R., unpublished). The effect of trypsin in preventing the increased localisation in CMI lesions was complete within the limits of detection (Tables 2, 3) and so the migration of both small and large lymphocytes was probably impaired. Second, large lymphocytes not only migrate preferentially into inflamed tissues but seem to be superior in migrating into non-lymphoid tissues in physiological conditions. This was suggested by injecting alternately labelled large and small lymphocytes and examining the cells migrating into normal rat skin¹³ and non-inflamed peritoneal cavity (O. Braendstrup and O. Werdelin, unpublished). In conclusion, although it is possible that trypsin may affect lymphocyte subsets differentially, it seems improbable that this factor could wholly explain the present results; at least some lymphocytes are capable of leaving the blood by either of two mechanisms.

In an adjuvant granuloma in sheep, the increased flux of lymphocytes was associated with a change in the appearance of the endothelium of small venules, which came to resemble the morphologically distinct high-walled endothelium in lymph nodes⁹. This is consistent with the notion that a granuloma accepts an increased influx of lymphocytes by the same mechanism as lymph nodes accept more lymphocytes from the blood than do non-lymphoid tissues. Of the two examples of a trypsin-sensitive mechanism of selective lymphocyte migration, the endothelial change in CMI may have appeared earlier in evolution since histologically well developed lymph nodes are confined to mammals. Possibly lymph nodes developed when the endothelium of small vessels in certain regions displayed as a permanent feature an adaptation which was previously unique to sites of chronic inflammation. Other features of lymph node organisation may have ensued as a result of the vascular change.

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Steroid-nucleosides possible novel agents for cancer chemotherapy

THE major classes of anti-cancer chemotherapeutic drugs include alkylating agents, anti-metabolites of nucleic acids, steroid hormones and antibiotics¹. Because of the lack of specificity of anti-tumour agents, attempts have been made to increase their specificity and cell-penetrating properties by coupling them to steroid hormones^{2,3}. In particular, steroidal alkylating agents exhibit carcinostatic properties if used in combination with other modes of therapy⁴. We have prepared a novel class of agents by coupling steroids with naturally occurring purines and pyrimidines by way of a C-N linkage. The structures are unique in that they may be regarded as analogues both of steroid hormones and of 'nucleosides' in which the sugar has been replaced. Accordingly, we may expect their steroid moiety to provide target specificity, while their nucleoside characteristics may result in interference at the DNA level to give the desired anti-tumour activity. Preliminary studies on the effect of steroid-nucleosides on the lifespan of tumour-bearing animals show that selectively coupled products exhibit anti-tumour activity.

The chemical combination of cholesterol, pregnenolone, progesterone and cortisone with various nucleoside bases and imidazole-derived heterocyclic compounds was accomplished by adapting general methods used in nucleoside synthesis⁵. Briefly, the appropriate bromosteroid was reacted with the persilylated heterocyclic in the presence of an HgO/HgBr_2 catalyst to give a C-N linkage. (Details of these preparations will be presented elsewhere.)

A number of our newly-synthesised steroid-nucleosides were tested for acute toxicity on CD-1 outbred albino female mice at 100 and 200 mg kg⁻¹ single dose levels (intraperitoneally, 0.2 ml sesame oil). None of these compounds proved to be toxic at these levels, with the exception of the 3 α -imidazole derivative of pregnenolone. This compound induced convulsions within 1 h at the lower concentration, and death within 12 h at the higher dose. A series of thymine and uracil derivatives were tested for anti-tumour activities on Fisher 344/CRBL female rats bearing the 13762 mammary adenocarcinoma. This tumour is sensitive to oestrogens and androgens. Progesterone does not significantly inhibit tumour growth⁶, however, even at

dose levels 15 times higher than those used in our screening (D. J. Taylor, personal communication). Accordingly, it is unlikely that any anti-tumour effects of our steroid-nucleosides could result from breakdown of the drugs to give the free steroid. The compounds selected for testing included the N_1 -thymine derivatives at the 3α -position of cholesterol (1), the 3α - and 3β -positions of pregnenolone (2) and (3), and the 21-position of progesterone (4); and the N_1 - and N_3 -uracil and N_1 -5-fluorouracil derivatives at the 3α -position of pregnenolone (5), (6) and (7). The effect on the lifespan of the animals is summarised in Table 1. Control animals treated with sesame oil alone developed large tumour masses and had a mean survival of 29.1 d. In the thymine series ((1)–(4)) where the compounds differed only in the steroid moiety, the 21-progesterone analogue (4) increased the mean survival time of the host to 45.7 d, thus exhibiting marked anti-tumour activity. The remaining compounds of this series showed only minimal effect. The 3α -pregnenolone-uracil compounds ((5)–(7)) differed in the site of attachment to the base, and although the N_1 -derivatives of uracil and of 5-fluorouracil were inactive, the N_3 -derivative of uracil (6) increased the mean survival time considerably, to 49.5 d. Treatment with 3α -imidazolepregnenolone (8) also prolonged the survival to 40.8 d.

Table 1 Mean survival times of Fisher 344/CRBL female rats bearing the hormone-sensitive 13762 mammary adenocarcinoma on treatment with a series of steroid-nucleosides

No.	Compound	Base-steroid	C-N linkage	Survival time d ($\pm$ s.e.)	Significance <i>P</i>
1	Thymine-cholesterol		3α (N_1)	29.1($\pm$ 2.8)	—
2	Thymine-pregnenolone		3α (N_1)	34.9($\pm$ 3.0)	0.2
3	Thymine-pregnenolone		3β (N_1)	37.3($\pm$ 3.8)	0.1
4	Thymine-progesterone		21 (N_1)	45.7($\pm$ 6.2)	0.02
5	Uracil-pregnenolone		3α (N_1)	31.6($\pm$ 0.8)	—
6	Uracil-pregnenolone		3α (N_3)	49.5($\pm$ 8.1)	0.01
7	5-Fluorouracil-pregnenolone		3α (N_1)	31.2($\pm$ 1.9)	—
8	Imidazole-pregnenolone		3α (N_1)	40.8($\pm$ 2.2)	0.001
0	Control			29.6($\pm$ 1.8)	—

Treatment was started 24 h after animals were given subcutaneous tumour grafts. The agents were administered subcutaneously in sesame oil (10 mg ml^{-1}) at a dose of 10 mg kg^{-1} on alternate days for a total period of 18 d. Groups of 10 animals were used (except for compound (4) which involved seven animals). Of the group treated with compound (6), four animals did not develop a palpable tumour. These four survivors were not considered in the determination of the standard error and significance. The level of significance (*P*) was evaluated using Student's *t* test. Progesterone itself does not affect this tumour⁶.

The data suggest that greater anti-tumour activity results from an N_3 -substitution of the pyrimidine and from the use of steroids with higher hormonal activity. Most conventional drugs have undesirable side effects due to their inhibiting effect on both normal and tumour cell proliferation, and must be given at high concentration to eradicate all growing tumour cells. Our derivatives are less likely to have adverse side effects, as their decomposition products are natural steroids and nucleoside bases. Based on these encouraging results and the above considerations we are pursuing a program of selective synthesis and more detailed biological evaluation of steroid-nucleosides.

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Glucocorticoid receptors and glucocorticoid sensitivity of mitogen stimulated and unstimulated human lymphocytes

GLUCOCORTICOIDS exert widespread suppressive effects on lymphocytes of most species^{1,2}. Sensitivity to the hormones is thought to vary with the state of lymphocyte maturation and differentiation. Immature thymic lymphocytes, for example, are more sensitive than mature thymic-dependent lymphocytes^{1,3}. Furthermore, *in vitro* studies have suggested that glucocorticoid sensitivity varies with the state of mitogen or antigen-induced activation. Nowell⁴ observed that prednisolone-21-phosphate reduced the number of mitoses induced in human peripheral blood lymphocytes by phytohaemagglutinin (PHA), and noted that this inhibition required the presence of glucocorticoids during the first 24 h of exposure to PHA. When glucocorticoids were added 48 h after PHA, a time when mitoses had not yet appeared, they had no effect on the number of mitoses measured 2 d later. Tormey *et al.*⁵ extended these results to show that prednisolone-21-phosphate inhibited incorporation of radiolabelled thymidine and cytidine into PHA-stimulated lymphocytes, and found that delayed addition of steroid resulted in diminished inhibition.

These and similar observations have led to the generally accepted view, basic to the application of glucocorticoids as immunosuppressants, that glucocorticoid sensitivity varies with the state of immunologic activation of the cell. Lymphocytes are thought to be insensitive until they are stimulated by specific antigen or mitogen, which induces an early glucocorticoid-sensitive phase before resistance again supervenes with full morphologic blast transformation and mitosis^{1,6}.

Because of the central role of glucocorticoid receptors in the action of glucocorticoids on lymphoid and other tissues, we have measured simultaneously the inhibitory effects of glucocorticoids on metabolic parameters and the number of glucocorticoid receptors in cultured human lymphocytes undergoing transformation. Our results indicate that, contrary to widespread assumption, lymphocytes are sensitive to metabolic inhibition regardless of their state of activation, and that furthermore, blast transformation is associated with a striking increase in the number of receptor sites per cell.

Human lymphocytes were cultured and assayed with dexamethasone (DX) for nuclear glucocorticoid receptor sites at 37 °C as described in the legend to Fig. 1. In this experiment, following addition of concanavalin A (Con A) to cultured lymphocytes, a sixfold increase in receptor sites per cell was noted by 24 h, with maximum levels occurring before 48 h (top curve, Fig. 1). By 6 d, levels returned to

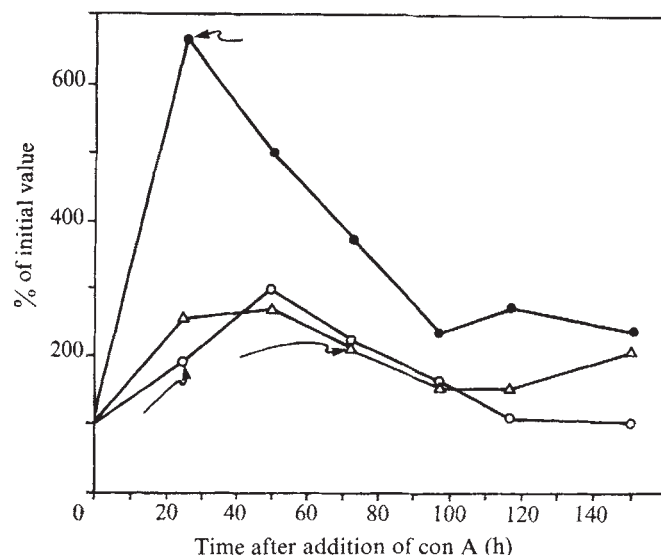


Fig. 1 Nuclear dexamethasone binding in human peripheral lymphocytes after Con A stimulation. Mononuclear cells were obtained from defibrinated fresh human blood by discontinuous density gradient separation over Ficoll-Hypaque¹⁰. Monocytes were removed by incubation on plastic for 18 h leaving a cell population consisting of 94% or more lymphocytes. Cells were cultured at 1×10^6 per ml in RPMI 1640 with 50 units per ml penicillin, $50 \mu\text{g ml}^{-1}$ streptomycin, 300 mM glutamine and 10% foetal calf serum. At time zero, $20 \mu\text{g ml}^{-1}$ con A was added. At the indicated times, aliquots containing 5×10^7 cells were removed and incubated at 37°C for 20 minutes (sufficient time to approach a steady state) with ^3H -DX (New England Nuclear; $22.6 \text{ Ci mmol}^{-1}$), at seven concentrations from $2 \times 10^{-7} \text{ M}$ to $5 \times 10^{-10} \text{ M}$. Nuclear pellets were obtained by lysis for measuring nuclear binding as described previously⁷. Results were plotted according to the method of Scatchard¹¹ to obtain estimates of the association constant and concentration of nuclear binding sites. Cell protein content was determined by the method of Lowry¹² using the supernatant after a freeze-thaw, hypotonic lysis, and cell volumes were calculated from the cell counts and the cytocrit (obtained by a micro haematocrit method) of the original cell suspension. Viability was determined from the proportion of cells excluding Trypan blue and was greater than 90% at the time of the assay. The 100% values are those for time equal to 0 h. ●, Sites per cell, 100% = 4,470. Δ, Sites per μg protein, 100% = 2.2×10^8 . ○, Moles per l cell volume, 100% = 1.5×10^{-8} .

approximately twice the initial value. Similar, but less pronounced changes were seen when results were expressed as binding sites per μg protein or per l cell volume (lower curves, Fig. 1). The exact time course of these changes was somewhat variable. In some experiments the peak number of binding sites did not occur until 72 h (for example Fig. 2). No significant changes were observed in the association constants, which averaged $1.4 \times 10^8 \text{ M}^{-1}$. Cytoplasmic receptor sites (data not shown), measured as previously described⁷, showed similar changes in number.

In separate experiments with cells from ten individuals (Table 1) we found a two–fourfold increase in DX binding sites per cell over unstimulated controls. This increase occurred regardless of the mitogenic stimulus. The controls showed no significant change in glucocorticoid binding.

Note that there was considerable variability in the receptor content of unstimulated cells. Cytoplasmic-to-nuclear translocation⁷ of the steroid receptor complex was found to take place normally in both mitogen-stimulated and control lymphocytes. When incubated with DX at 3°C , most of the binding was restricted to the cytoplasm, but after warming the cells to 37°C for 20 min, 50–80% of the complexes became nuclear bound. Cortisol, corticosterone and corticosterone at 10^{-6} M competed significantly for binding of DX in both stimulated and unstimulated lymphocytes. Cortisone, testosterone, estradiol and progesterone at 10^{-6} M did not show significant competition. The time course of nuclear binding at 37°C reached a steady state after 20 min of exposure to DX for both control and con A-stimulated lymphocytes. Thus the DX-binding substances in mitogen-stimulated lymphocytes behave in all respects like glucocorticoid receptors in normal lymphocytes⁷, differing from those in the unstimulated cells only by their greater number.

The sensitivity of the Con A-stimulated lymphocytes to metabolic inhibition by DX at various times after activation is shown in Fig. 2. DX (10^{-7} M) was added after 24, 48 and 72 h of culture in the presence of con A. After 6 and 19 h exposure to DX, the incorporation of tritiated thymidine (^3H -Tdr) and tritiated uridine (^3H -Urd) was measured. The number of DX binding sites after exposure to con A was determined on separate aliquots, as described in Fig. 1. DX produced significant suppression of the incorporation of both the isotopes, but did not alter viable cell numbers. The degree of suppression of isotope incorporation did not vary significantly with time of exposure to Con A was determined on separate aliquots, as described increased fourfold during the culture period. We have also conducted these experiments with 10^{-6} , 10^{-8} and 10^{-9} M DX, with essentially the same results as with 10^{-7} M , except that suppression was dose-dependent and marginal with 10^{-9} M .

Similar studies (results not shown) were performed using unstimulated human lymphocytes placed into culture with and without DX (10^{-10} to 10^{-6} M). In five experiments there was a dose-dependent suppression of ^3H -Urd and ^{14}C -leucine incorporation of similar magnitude to the suppression shown in Fig. 2, measured after 24, 48 and 72 h of culture.

DX decreased glucose uptake by approximately the same absolute amount in both con A-stimulated and unstimulated lymphocytes (Fig. 3). The relative decrease was less for the stimulated cells, because of their higher base level of glucose uptake.

These studies demonstrate that mitogen stimulation leads to a marked increase in the number of glucocorticoid receptors per cell and that the cells remain sensitive to glucocorticoids. Our results do not necessarily contradict the earlier observations cited above. Previous investigators have generally measured the number of mitoses, number of blast transformed cells, or isotope incorporation at a fixed time after addition of mitogen with a varying time of exposure to glucocorticoids^{4,5}. These experimental approaches create an apparent loss of suppressive effect if the suppressive agent is added after the cells have begun to

Table 1 Effects of mitogenic stimuli on nuclear dexamethasone binding sites

Stimulus	No. of experiments	Unstimulated	Dexamethasone binding sites per cell Stimulated	Stimulated/unstimulated
Con A	7	$3151 \pm 726^*$	$9,483 \pm 1,513^*$	$3.79 \pm 0.71^*$
PHA	1	1640	10,300	6.28
PWM	1	2490	6,750	2.71
MLC	2	1,541; 4,070†	3,248; 11,160†	2.11, 2.71†

Human lymphocytes were obtained and nuclear binding of DX determined as in the legend for Fig. 1. Cells were stimulated by incubation with con A $20 \mu\text{g ml}^{-1}$ for 3 d, PHA $10 \mu\text{g ml}^{-1}$ for 3 d and PWM (Pokeweed mitogen, GIBCO) for 5 d. Lymphocytes stimulated by mixed lymphocyte culture (MLC) (ref. 13) were obtained after 7 d incubation with allogeneic irradiated lymphocytes.

*Mean $\pm$ s.e.m.

†Values shown are ranges for the 1st and 2nd subject respectively.

proliferate in an exponential fashion. To accurately determine sensitivity, cells must be allowed to pass through blast transformation and equal numbers of cells, at the same stage of activation, must be compared with and without the suppressive agent. As we have shown using this experimental approach, human lymphocytes are equally sensitive to glucocorticoid suppression at all stages of activation, despite large increases in receptor number.

The increase in receptor number which follows mitogen stimulation may reflect variations in receptor number with phases of the cell cycle, a degree of synchronisation having been achieved by the sudden stimulation with mitogen. Cidlowski and Michaels⁸ have found that in synchronised HeLa cell cultures there is a doubling of receptor number per cell as the cells pass through late G₁, followed by return to the original number after mitosis.

The finding of an increased number of receptors in blast transformed cells, and the fact that receptor number does not relate directly to the level of glucocorticoid effect, raises practical considerations in the evaluation of glucocorticoid receptors and sensitivity of malignant lymphocytes. Lippman *et al.*⁹ have reported that lymphoblasts from patients with acute lymphoblastic leukaemia have higher levels of receptors than normal peripheral lymphocytes.

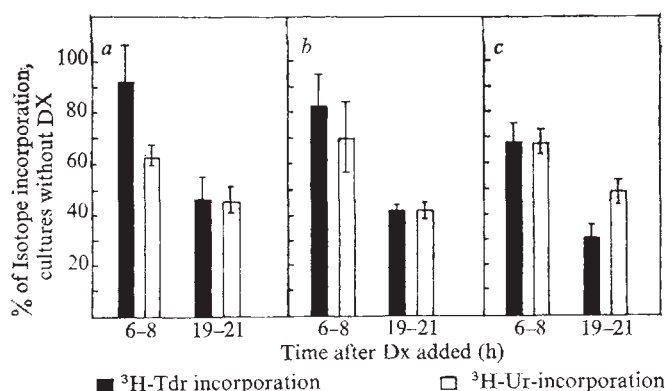


Fig. 2 Relationships between con A-induced blastogenesis, glucocorticoid receptor sites and DX suppression of tritiated thymidine and tritiated uridine incorporation. Lymphocytes (1×10^6 cells per ml) were cultured in RPMI 1640 medium, 10% autologous plasma, penicillin G, 50 units per ml, and streptomycin 50 $\mu\text{g ml}^{-1}$. Con A (20 $\mu\text{g ml}^{-1}$) was added as a mitogenic stimulus. Cells were collected after 24, 48 and 72 h, and washed twice with Hanks balanced salt solution. The number of glucocorticoid receptor sites was determined as described in Fig. 1. The percentage of lymphoblastoid cells was determined on Wright-Geimsa stained Cytospin (Shandon-Southern Instruments) preparations. Cells were scored as blastoid if they were large with light staining nuclei, prominent nucleoli, and increased basophilia of the cytoplasm. Five hundred cells were counted. Aliquots of these cells (1×10^5 in triplicate) were placed into microtest-II plates (Falcon) with and without DX (10^{-7} M). Tritiated thymidine (2 $\mu\text{Ci ml}^{-1}$; 1.9 Ci mmol $^{-1}$) or tritiated uridine (2 $\mu\text{Ci ml}^{-1}$; 1.9 Ci mmol $^{-1}$) were added to the cultures from 6 to 8 and 19 to 21 h after addition of DX. Cultures were harvested on to glass fibre filters by means of a multiple automated sample harvester, washed with distilled water¹³, dried and counted in a liquid scintillation counter. Results are expressed as a percentage of isotope incorporation of cultures without dexamethasone. Brackets represent one standard deviation of per cent control. Viable cell numbers were determined by Trypan blue dye exclusion and Model B Coulter Counter before addition of DX and at the time of cell harvest. There was no significant change in cell number. The 100% values expressed as c.p.m. of isotope incorporated ± 1 s.d. were as follows: at 24 h, 6-8-h pulse $^3\text{H-Tdr} = 44 \pm 6$, $^3\text{H-Ur} = 574 \pm 35$, 19-21-h pulse $^3\text{H-Tdr} = 1109 \pm 169$, $^3\text{H-Ur} = 905 \pm 105$; at 48 h, 6-8-h pulse, $^3\text{H-Tdr} = 2674 \pm 238$, $^3\text{H-Ur} = 2511 \pm 274$, 19-21-h pulse, $^3\text{H-Tdr} = 2494 \pm 334$, $^3\text{H-Ur} = 1442 \pm 39$; at 72 h, 6-8-h pulse, $^3\text{H-Tdr} = 2710 \pm 131$, $^3\text{H-Ur} = 1313 \pm 72$, 19-21-h pulse, $^3\text{H-Tdr} = 768 \pm 123$, $^3\text{H-Ur} = 335 \pm 27$. a, Con A present for 24 h; 44% blastoid cells; 4,190 binding sites per cell. b, Con A present 48 h; 55% blastoid cells. c, Con A present 72 h; 76% blastoid cells; 16,775 binding sites per cell. Shaded bars, $^3\text{H-Tdr}$ incorporation; open bars $^3\text{H-Ur}$ incorporation.

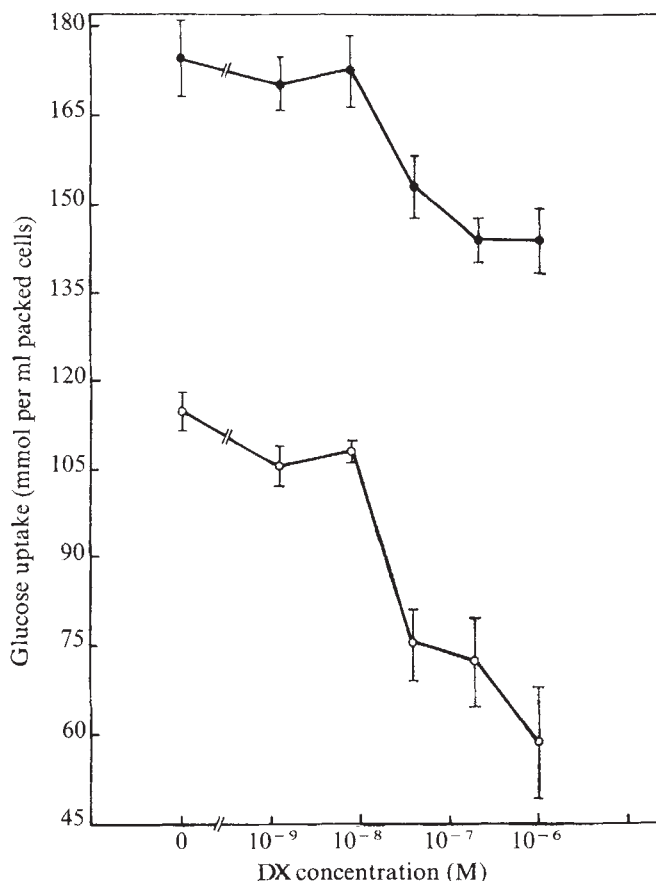


Fig. 3 Effect of DX on glucose uptake by unstimulated and con A-stimulated human peripheral lymphocytes. Lymphocytes cultured as described for Fig. 1 in the presence and absence of con A for 3 d, were washed and resuspended at 7×10^6 per ml in Krebs Ringer bicarbonate buffer without glucose (gassed with 5% CO₂, 95% air). At time zero, 20 μl of the cell suspension was added to 20 μl of similar medium containing 4.4 mM glucose with varying concentrations of DX and incubated in wells of V-bottom microtitre plates (Linbro) for 5 h at 37 °C with 5% CO₂ in humidified air without shaking. At the end of the incubation glucose concentration in the supernatant was measured by the glucose oxidase method (Worthington). The points represent means and vertical bars represent one standard error of four replicates. ○, Unstimulated lymphocytes; ●, con A-stimulated lymphocytes.

These findings have recently been extended to include lymphoblast cells from patients with Burkitt's lymphoma and poorly differentiated lymphocytic lymphoma (G. Konior, personal communication). These investigators did not explore the glucocorticoid sensitivity of the cells as it related to receptor number, but speculated that a low receptor number might correlate with glucocorticoid insensitivity. Our results suggest that in human lymphoid cells, receptor number may correlate with the proliferative stage of the cell and may not determine glucocorticoid sensitivity.

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MSA and EGF receptors on sarcoma virus transformed cells and human fibrosarcoma cells in culture

CULTURED cells transformed by murine or feline sarcoma viruses lose their ability to bind epidermal growth factor (EGF) to cell surface receptors, whereas cells transformed by DNA tumour viruses or infected with non-transforming RNA viruses show normal EGF binding¹. We demonstrate here that this sarcoma virus-dependent receptor loss is specific for EGF. The binding of a different polypeptide growth factor, multiplication stimulating activity²⁻⁴ (MSA), to its cell surface receptors was not decreased in murine and feline sarcoma virus transformed cells. In two cell lines established from human fibrosarcomas, however, MSA binding was abolished while EGF bound to the same extent as to normal human fibroblasts.

MSA is the name given to a family of polypeptides of molecular weight approximately 10,000 that is synthesised by a line of Buffalo rat liver cells in culture²⁻⁴. Like EGF and fibroblast growth factor⁵ (FGF), MSA is an efficient stimulator of cell DNA synthesis in chick embryo², rat³, 3T3 mouse⁶ and human⁷ fibroblasts in culture, and in a rat muscle cell line⁸. Specific receptors for MSA have been characterised in rat liver plasma membranes^{9,10}, and in cultures of chick embryo fibroblasts^{9,11}, adult human skin fibroblasts⁷, and a subclone of the rat liver cell line that produces MSA⁴. The binding of ¹²⁵I-labelled MSA to these receptors was inhibited only by MSA itself and by two polypeptides purified from human plasma, somatomedin A and non-suppressible insulin-like activity^{7,10,11} (NSILA-s); ¹²⁵I-MSA binding to chick embryo and human fibroblasts also was inhibited by insulin and proinsulin^{7,9,11}. Other peptide hormones and growth factors did not compete for MSA binding.

We studied MSA binding in normal rat kidney (NRK clone 2) and mink lung cells, and their sarcoma virus transformants (Table 1). The conditions of the receptor assays were those previously used to study the binding of type C

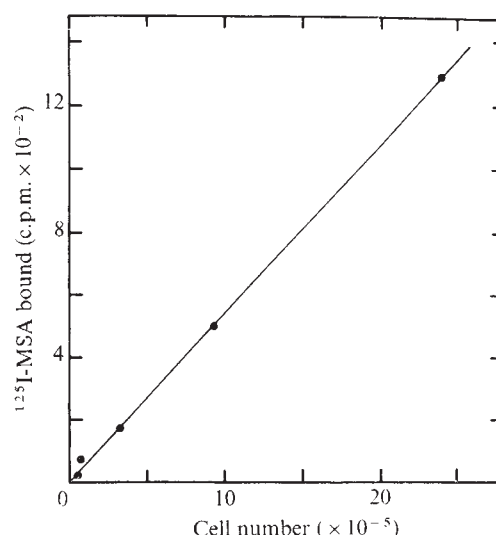


Fig. 1 ¹²⁵I-MSA binding as a function of cell number. ¹²⁵I-MSA was incubated with NRK clone 2 cells at the indicated densities per 60-mm plate. The incubations were performed as described in Table 1. The specific binding of ¹²⁵I-MSA is plotted as a function of the cell number.

viral envelope glycoprotein¹² and EGF¹ to cellular receptors, with the exception that the incubations were performed at 22 °C. Subconfluent cell monolayers were used so that both normal and transformed cells would be actively growing. MSA binding increased linearly with the number of NRK cells seeded per 60-mm Petri plate over the range 2.7×10^4 to 2.4×10^6 cells (Fig. 1). Although significant MSA binding can be detected with as few as 5×10^4 NRK cells, most experiments were performed with 0.5 – 1.0×10^6 cells per plate.

Normal rat and mink cells have different receptors that bind MSA or EGF selectively (Table 1, Fig. 2). The binding of ¹²⁵I-MSA was inhibited by unlabelled MSA, with 50% of the maximal inhibition by 133 ng ml^{-1} . In contrast, EGF, fibroblast growth factor, nerve growth factor, and insulin at $6.7 \text{ } \mu\text{g ml}^{-1}$ did not inhibit ¹²⁵I-MSA binding. Approximately 90% of total ¹²⁵I-MSA binding could be abolished by incubation with $3.3 \text{ } \mu\text{g ml}^{-1}$ MSA, and is referred to as specific binding. ¹²⁵I-EGF also bound specifically to normal rat and mink cells. Its binding was inhibited by EGF, but was not affected by high concentrations of MSA.

Table 1 ¹²⁵I-EGF and ¹²⁵I-MSA binding to normal and transformed cells

¹²⁵ I-labelled	Ligand	Unlabelled	Normal rat kidney cells		Normal mink lung cells		FeSV transformed
			Control	K-MSV transformed	Control	K-MSV transformed	
EGF	None		1,970	120	9,430	320	440
EGF	EGF		170	180	190	190	150
EGF	MSA		1,400	120	9,700	260	420
MSA	None		1,445	930	1,023	1,090	1,145
MSA	MSA		120	135	131	175	110
MSA	EGF		1,945	925	1,156	1,010	1,215

¹²⁵I-EGF and ¹²⁵I-MSA binding to normal cells and cells transformed by Kirsten murine sarcoma virus (K-MSV) and feline sarcoma virus (FeSV). EGF was prepared from male mouse submaxillary glands by acid extraction followed by gel filtration and DEAE-cellulose chromatography¹³. MSA was purified from the serum-free conditioned media obtained from a rat liver cell line (BRL3A) in culture^{2,3}. Purification procedures included ion exchange chromatography through Dowex-50, gel filtration chromatography on Sephadex G-50 at acid pH, and preparative acrylamide electrophoresis⁴. The iodination of EGF²⁰ and MSA^{9,11,12} binding assay procedures and cell designations have been previously described¹. Cells were plated in 60-mm plastic Petri dishes (Falcon) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum at the indicated densities: control normal rat kidney, NRK clone 2 (5×10^5 cells per dish); K-MSV transformed NRK (5×10^5 cells per dish); control normal mink lung cell (7×10^5 cells per dish); K-MSV transformed mink lung cell (6×10^5 cells per dish); and FeSV transformed mink cell (6×10^5 cells per dish). After 18 h the plates were washed twice with 1.5 ml of binding buffer (DMEM containing 1 mg ml⁻¹ bovine serum albumin and 50 mM N,N-bis-(2-hydroxyethyl)-2-aminoethane-sulphonic acid (BES), pH 6.8). Binding reactions were initiated by the addition of 1.5 ml of binding buffer containing either 3 ng of ¹²⁵I-EGF (120,000 c.p.m.) or 0.4 ng of ¹²⁵I-MSA (34,200 c.p.m.) with or without 5 μg of unlabelled EGF or MSA. The dishes were incubated at 22 °C for 90 min. At the end of the incubation, the media was removed and the cell monolayers were washed four times with cold binding buffer to remove the unbound ¹²⁵I-peptides. The amount of ¹²⁵I-peptide bound to the cells was measured after lysing the cells. The radioactivity present in the lysate¹ was determined using a Beckman LS-250 liquid scintillation counter at 60% efficiency.

Transformation of rat and mink cells with either murine or feline sarcoma viruses abolished approximately 90% of the specific EGF binding, but did not significantly reduce MSA binding (Table 1). Similar results were obtained when the binding of MSA and EGF to normal mink lung cells and mink lung cells transformed by murine sarcoma virus was compared after incubation for different times ranging from 15–180 min (Figs 3*a, b*). These results confirm that sarcoma virus transformation selectively perturbs the EGF receptor system.

We next asked whether the ability to bind MSA might be lost in other types of transformed cells. Mouse and rat clones transformed by the DNA viruses SV40, polyoma, and adenovirus, as well as a series of chemical carcinogen-transformed mouse clones were examined. In 17 of 18 clones tested MSA was bound to a similar extent in the transformed and parental cell lines. One clone of polyoma-transformed 3T3, PY-3T3-4A (ref. 13), has consistently shown low levels of MSA binding (10–20% of the control 3T3 cells), but three other independently isolated polyoma-transformed 3T3 clones showed normal levels of MSA binding. Transformation of cells in culture, then, is apparently not associated with a specific alteration of MSA receptors.

In striking contrast is the selective loss of MSA binding to two cell lines that have been established from human fibrosarcomas (Figs 3*c, d*). These lines, 8387 (ref. 14) and HT1080 (ref. 15) have been previously characterised. Line 8387 was derived from a 25-yr-old female with fibrosarcoma of the leg; HT1080 was from a 35-yr-old male with a fibrosarcoma of the acetabulum. Less than 5% as much ^{125}I -MSA was bound by the 8387 and HT1080 fibrosarcoma cell lines than by foetal and newborn fibroblasts. In contrast, ^{125}I -EGF bound to comparable extents and with the same kinetics to the same fibrosarcoma cells and to embryonic and newborn human fibroblasts. The low binding of ^{125}I -MSA to the fibrosarcoma cells did not result from an altered kinetics of binding (Figs 3*c, d*). Neither did it seem to result from degradation of ^{125}I -MSA by the fibrosarcoma cells. Pre-incubation of ^{125}I -MSA with 8387 cells for 2 h

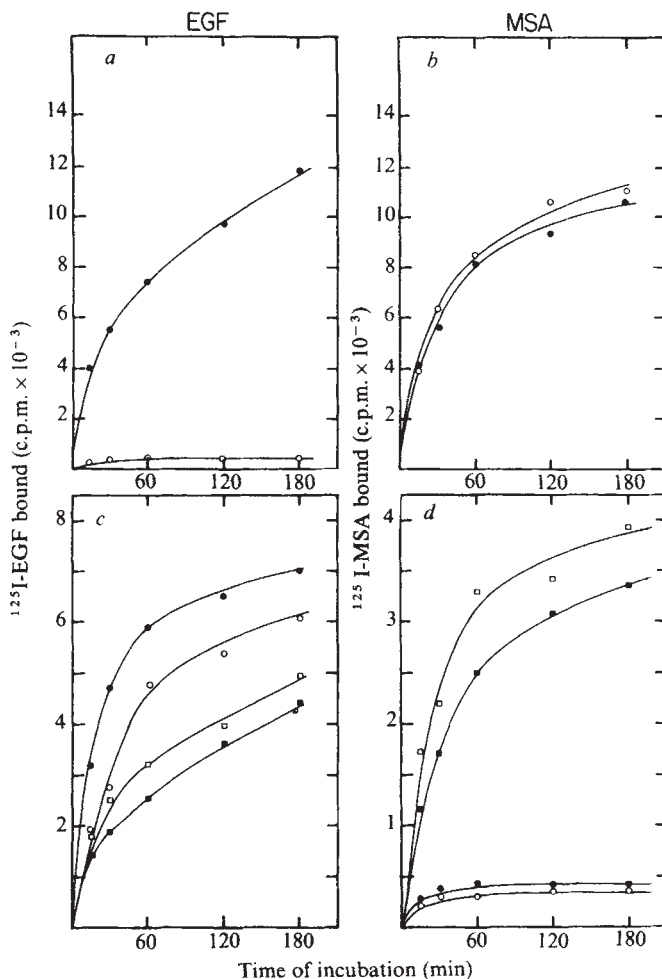


Fig. 3 Time course of association of ^{125}I -EGF (*a, c*) and ^{125}I -MSA (*b, d*) to different cell types. *a, b*: ^{125}I -EGF (*a*) or ^{125}I -MSA (*b*) were incubated with normal mink cells (●, 7×10^5 cells per dish) or Kirsten sarcoma virus transformed mink cells (○, 6×10^5 cells per dish). *c, d*: ^{125}I -EGF (*c*) or ^{125}I -MSA (*d*) were incubated with four human lines or strains: 8387, a human fibrosarcoma line (●, 7×10^5 cells per dish); HT1080, another human fibrosarcoma line (○, 5×10^5 cells per dish); HuF, a human foreskin fibroblast strain (□, 4×10^5 cells per dish) W138, a human embryonic lung fibroblast strain (■, 4×10^5 cells per dish). The data are not corrected for counter background (~ 60 c.p.m.) nor nonspecific binding (~ 250 c.p.m. for ^{125}I -MSA).

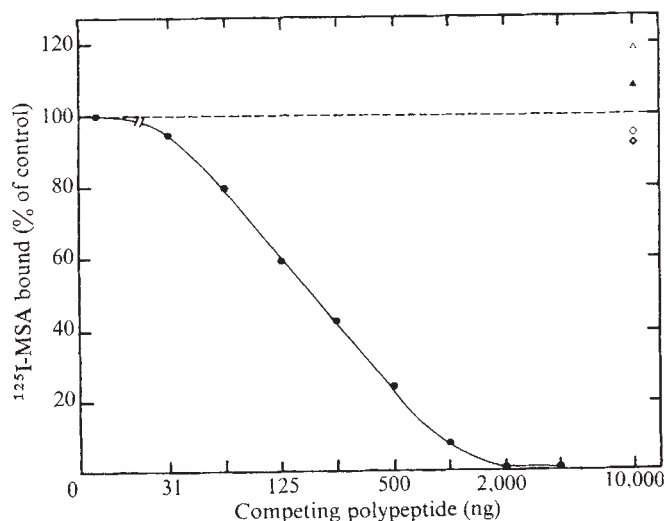


Fig. 2 Competition for ^{125}I -MSA binding by unlabelled peptides. ^{125}I -MSA (0.2 ng ml^{-1} ; $14,000$ c.p.m.) was incubated with NRK clone 2 cells (9.5×10^5) for 90 min at 22°C in the presence of varying amounts of unlabelled peptides. MSA, ●; EGF, △; nerve growth factor, ▲ (gift from R. Levi-Montalcini); fibroblast growth factor, ◆ (gift from D. Gospodarowicz); crystalline zinc porcine insulin, ◇ (Lilly). Nonspecific binding (the ^{125}I -bound ligand which could not be competed by a large excess of unlabelled ligand) has been subtracted. The data presented are the mean of duplicate determinations. The % of control binding (2.1% of input radioactivity) is plotted against the log of the unlabelled competing peptide.

did not destroy the ability of the ligand recovered from the incubation medium to bind to receptor-positive cells. Similarly, fibrosarcoma cells cocultivated at fiftyfold excess (2×10^6 cells per plate) with NRK clone 2 cells (4×10^4 cells per plate) did not inhibit ^{125}I -MSA binding to the rat cells.

The lack of MSA binding to the two human fibrosarcoma cell lines is especially distinctive because MSA binds well to fibroblasts and other mesenchymal cells from humans and other species, and to cell lines established from a variety of other human tumours. MSA receptors were found in 34 out of 34 fibroblast strains from 12 different species. More than 20 normal human fibroblast strains derived from fetuses, newborns, and adults of various ages showed more than 10 times greater MSA binding than that of the fibrosarcoma lines. Further, loss of MSA receptors is not a general property of human tumour cells in culture, since other human tumour lines including chondrosarcomas, glioblastomas, and renal cell carcinomas specifically bound labelled MSA (in preparation).

Since the progenitor cells of the fibrosarcomas presumably had MSA receptors, the selective loss or unavailability of MSA receptors in the fibrosarcoma cell lines suggests the possibility that an alteration in the MSA-

receptor system may have had a role in the cellular transformation process. It is intriguing that fibrosarcomas are among the human tumour types that have been associated with clinical hypoglycaemia not attributable to elevated plasma insulin levels¹⁶. Markedly elevated levels of NSILA-s, a polypeptide with weak insulin-like activity, were identified in the serum of one patient with a fibrosarcoma and hypoglycaemia; presumably the tumour was the source of the circulating NSILA-s¹⁷. NSILA-s and MSA are closely related peptides that interact with the same receptors^{4,7,10,11}. This raises the possibility that the fibrosarcoma cell lines produce MSA-like polypeptides. The endogenous peptide production might occupy receptors, or regulate their availability¹⁸. Experiments to test for production of these factors by the fibrosarcoma lines in culture are in progress.

Thus, the binding of two polypeptide growth factors, EGF and MSA, seems to be selectively altered in different types of cell transformation. MSA binding is markedly reduced in two human fibrosarcoma cell lines, whereas EGF binding is abolished by murine and feline sarcoma virus transformation. While the precise relationship between the availability of growth factor receptors and transformation is unknown, we propose the following model for consideration. Cell growth in an organism is controlled, in part, by the particular display of specific growth factor receptors responding to hormones produced by other cells. In the normal situation, those cells that produce a specific growth factor do not also respond to it. But, the inappropriate production by a target cell of an active hormone that it also has receptors for may be sufficient to serve as a stimulus to cell division. Persistent uncontrolled production of the growth factor may then serve as a continuous endogenous stimulus and lead to inappropriate cell growth.

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Simian virus 40 facilitates multiplication of replication defective mutants of polyoma virus in BALB/3T3 mouse cells

STUDIES on conditionally lethal mutants of polyoma virus or simian virus 40 (SV40) should provide information on viral gene functions in infected cells. Temperature-sensitive mutants of polyoma virus^{1–4} and SV40 (refs 5–8) and a host range mutant of polyoma virus^{9,10} have already been isolated. But, the functional relationship between the viral genes of polyoma virus and SV40 has not yet been studied. To examine the relationship between the viral gene functions of polyoma virus and SV40, and changes of the cellular regulation system in cells transformed by SV40, we attempted to isolate new host range mutants of polyoma virus which could replicate in mouse cells transformed by SV40 but not in normal mouse cells. We report here the isolation of two host range mutants of this type. Productive infection of these mutants in A31-714 cells¹¹ (subclone of mouse BALB/3T3), which are restrictive for these mutants, is facilitated by co-infection with SV40.

Host range mutants were isolated from ultraviolet-irradiated polyoma virus (strain: LP147-2). A sample of 2 ml of fluid containing polyoma virus was put into a Petri dish (40 mm in diameter) and irradiated for 3 min under a Toshiba germicidal lamp (15 W×2) from a distance of 15 cm with gentle shaking. In these conditions, the plaque-forming ability of the polyoma virus was reduced to 10⁻³ of the initial value. After irradiation, appropriate dilutions of the sample were plated on subconfluent monolayers of SA31 cells (A31-714 cells transformed by SV40 with the following properties; (1) SV40 T-antigen positive, (2) virus-free, (3) SV40 inducible by cell fusion with CV-1 cells by UV-HVJ, and (4) capable of use in plaque assay of polyoma virus). After 7–8 d single plaques were picked up and each was tested for its ability to produce plaques on confluent monolayers of A31 cells.

Two of 576 plaques tested failed to produce distinct plaques on A31 cells. The mutants obtained by three further sequential plaque purifications on SA31 were named HR-86 and HR-101.

Table 1 shows the abilities of mutant HR-101 to form plaques on monolayers of A31-714 cells and SA31 cells. The plaque forming ability of the wild type was the same on both indicator cells, whereas that of HR-101 was 10⁻²

Table 1 Plaque-forming abilities of HR-101 in A31-714 cells and SA31 cells

Type of virus	Type of cells	PFU per ml
HR-101	A31-714	< 10 ⁴
HR-101	SA31	3.1 × 10 ⁶
WT	A31-714	2.1 × 10 ⁶
WT	SA31	2.0 × 10 ⁶

Plaques were counted 8 d after virus infection. PFU, plaque-forming units.

or less on A31-714 cells than on SA31 cells. Cytopathogenic effects were seen, however, when low dilutions of HR-101 samples were inoculated on to monolayers of A31-714 cells.

To see whether the reduction in the plaque-forming ability of HR-101 in A31-714 cells was due to decrease in virus yield, the replications of the mutant in A31-714 cells and in SA31 cells were compared.

A31-714 cells and SA31 cells were both infected with HR-101 and 6 h later uninfected virus was washed out and production of virus was followed at 24-h intervals.

When HR-101 was inoculated on to A31-714 cells, it did not show appreciable multiplication, but when it was inoculated on to SA31 cells it multiplied: multiplication

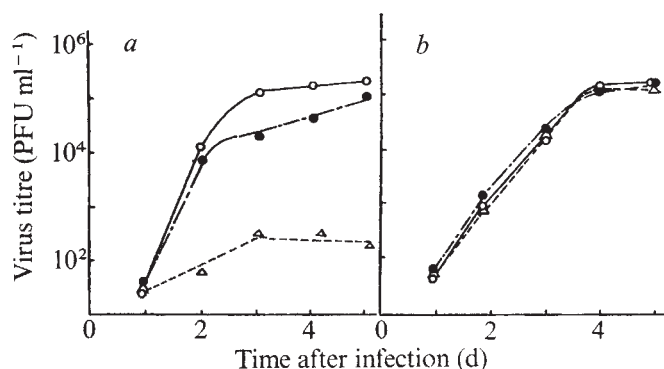


Fig. 1 Cultures of A31-714 and SA31 inoculated with HR-101, Wt, HR-101 plus SV40 (777 strain) or WT plus SV40. The multiplicities of infection of HR-101 and WT were 0.03 and that of SV40 was 60. After adsorption at 37 °C for 6 h, cultures were washed three times with medium, covered with 5 ml of Eagle's MEM medium containing 10% calf serum and incubated at 37 °C at 24 h intervals, cultures were collected and stored frozen. Cells and media were sonicated and their virus titres were assayed by plaque formation on cultures of SA31 cells. Growth of HR-101 (a) and WT (b) in SA31 cells (○), in A31-714 cells without SV40 (△) and in A31-714 cells with SV40 (●).

was detectable after 2 d and reached a maximum after 3–4 d (Fig. 1a). In contrast, the wild type multiplied equally well in A31-714 cells and SA31 cells (Fig. 1b).

To determine whether the change in susceptibility of A31-714 cells to infection by the mutant was caused by SV40, the helper function of SV40 in multiplication of the mutant in A31-714 cells was also examined. A31-714 cells were co-infected with HR-101 (multiplicity of infection (m.o.i.), 0.03) and SV40 (m.o.i., 60) and the yields of HR-101 were followed by the method described above.

The yield of HR-101 in A31 cells was enhanced approximately 60-fold by co-infection with SV40 (Fig. 1a).

When SV40 samples were treated with anti-SV40 rabbit serum, their ability to enhance the replication of HR-101 disappeared completely (Table 2). The viral characters of HR-86 were similar to those of HR-101 described above.

In preliminary experiments, cells in primary cultures from embryos of a BALB/c mouse also showed low susceptibility to host range mutants and co-infected SV40 enhanced the growth of these mutants in the primary cells (data not shown).

These results suggest that SV40 converts the susceptibility of mouse cells to infection with these mutants from a restrictive to a non-restrictive state. Although the role of SV40 in this conversion of cellular susceptibility has not yet been elucidated, the restrictive point to the infection of HR-101 in A31-714 cells may be considered not before the penetrat-

Table 2 Effect of anti-SV40 serum on the helper function of SV40

Type of infection	Type of cells	Virus titre per ml*	Enhancement ratio
HR-101	A31-714	7.8×10^2	1.0
HR-101 + SV40	A31-714	4.5×10^4	57.7
HR-101 + (SV40)†	A31-714	2.8×10^2	0.4
HR-101	SA31	1.0×10^5	128.2

Cultures of A31-714 and SA31 containing 3.2×10^5 cells per 4-cm plastic dish were inoculated with HR-101 at a multiplicity of 0.03. Some cultures of A31-714 were inoculated with both HR-101 and SV40 (m.o.i., 10) or HR-101 and SV40 which had been treated with anti-SV40 serum. After adsorption at 37 °C for 6 h, cultures were washed three times with medium and covered with 5 ml of Eagle's MEM medium containing 10% calf serum. These cultures were collected 72 h later. The cells and media were sonicated and virus infectivity in the lysates was assayed by plaque formation on cultures of SA31 cells.

*72 h post-infection.

†Treated with anti-SV40 serum.

ing step but after the uncoating step, because DNA from HR-101 was also considerably less infectious to A31-714 cells than to SA31 cells (Table 3). The defect in virus growth of the mutants may be helped either by the direct action of a SV40 viral gene product(s) present in both SV40-transformed cells and mouse cells abortively infected with SV40, or by the indirect action in which a SV40 genome in these cells alters the expression of cellular genes. In any case, an 'early' event(s) of SV40 infection may be concerned with change in susceptibility to these host range mutants because SV40 did not produce 'late' gene products in mouse cells on either infection or transformation^{12,13}.

Table 3 Virus yields after infection with viral DNA

DNA	DNase treatment (20 µg ml ⁻¹)	Yield (PFU per culture) A31	Yield (PFU per culture) SA31	Ratio SA31 : A31
Wt	—	1.5×10^3	1.8×10^3	1.2
	+	< 10	< 10	—
HR-101	—	5.0×10^1	9.0×10^3	180.0
	+	< 10	< 10	—

A confluent monolayer of A31-714 cells or SA31 cells in a 6-cm Petri dish was washed once with 5 ml of phosphate-buffered saline and then infected with 0.1 ml of a mixture containing wild-type DNA (7.2×10^2 PFU: assayed on SA31 cells) or HR-DNA (2.8×10^3 PFU: assayed on SA31 cells), DEAE-dextran (500 µg ml⁻¹) and Tris-HCl (pH 7.5, 0.05 M). After incubation at room temperature for 30 min, the cells were washed once and then 5 ml of growth medium (MEM + 10% calf serum) were added. After 3 d at 37 °C, cells and fluids were collected, sonicated and assayed on SA31 cells to determine virus yield.

Further experiments on the characteristics of host range mutants and on the relation between the gene functions of polyoma virus and SV40 are in progress.

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Extraneural localisation of herpes simplex virus in latently infected guinea pigs

CHRONIC latent herpes simplex virus (HSV) infections are associated with tissues of the nervous system in experimentally infected mice and rabbits^{1,2} as well as in humans^{3–5}. Cook and Stevens demonstrated, that, after inoculation into the skin, HSV migrates via the subserving nerves to the corresponding sensory ganglia⁶. The virus establishes there a latent infection which can be detected by methods of *in vitro* cultivation of the ganglia^{1–5}. A consideration of the data obtained so far on the behaviour of latent HSV in mice, rabbits and men led Cook and Stevens as well as Baringer to the postulate, that latent HSV infections were restricted to tissues of the nervous system^{7,8}. The failures to detect HSV in the skin during latent infections are con-

Table 1 Recovery of HSV type 2 during the chronic latent infection

Animal	Day after infection	Virus recovered from				Number of recurrent eruptions	Day after infection when last recurrence developed
		Footpad	Sciatic nerve	Dorsal root ganglia (L ₄ -S ₃)	Lumbosacral spinal cord		
261	97	+	+	+	ND	None	—
885	97	+	+	+	ND	1	41
298	97	+	—	—	ND	None	—
274	133	+	—	+	—	2	73
839	136	+	—	+	—	2	80
569	136	+	—	+	—	4	104
272	143	+	—	+	—	None	—
241	143	—	+	—	—	None	—
775	194	+	—	—	+	2	125
88	194	+	—	+	—	3	38
230	208	+	+	—	+	1	38
757	208	+	?	—	+	1	73
799	210	+	+	—	ND	4	110
883	210	+	+	+	ND	2	95
603	210	+	+	—	ND	2	58

ND, not done. ?, Cultures contaminated.

sistent with this hypothesis^{9,10}. Recurrent HSV infections are thought to arise by activation of the latent infection within the ganglia and the subsequent migration of the reactivated virus into the skin via the corresponding nerve fibres^{9,11}. I have described recently experimental infections of guinea pigs with HSV type 2, which led to chronic latent infections of the animals with frequent spontaneously recurring lesions at the sites of initial inoculation^{12,13}. In this report I present evidence that during the latent phase of the infection HSV can be recovered not only from nervous tissues but also from the skin at the site of the primary infection.

Fifteen albino guinea pigs were inoculated with 10⁴ plaque-forming units of HSV type 2, strain 72, subcutaneously into the left hind footpad. They were observed for clinical signs of primary and recurrent herpetic lesions three times a week. The animals were sacrificed between 3 and 7 months after infection at times when they did not show any clinical sign of recurrent infection. Pertinent tissues were taken from exsanguinated animals and assayed for latent virus by explantation on monolayers of primary rabbit kidney (PRK) cells as described previously¹¹. All isolates were passaged once on PRK cells and identified as HSV by neutralisation with an anti-HSV hyperimmune serum, prepared in rabbits.

The data (Table 1) show, that all 15 animals harboured latent HSV, irrespective of whether they had developed previous recurrent infections or not. Surprisingly, however, from 14 of the animals virus was isolated from the skin of the initially infected footpad, whereas only eight animals were shown to harbour virus in their lumbosacral dorsal root ganglia and three animals in the lumbosacral part of the spinal cord. In addition, HSV was recovered from the sciatic nerve trunks of seven animals.

To test whether chronic latent infections of guinea pigs could be established with HSV type 1 as well and if so, where the virus would be located, a group of animals was similarly infected with HSV type 1, strain McIntyre. This strain does not induce recurrent infections in guinea pigs (unpublished). Chronic latent infection of the skin of the footpad was detected in six out of eight animals (Table 2).

None of the eight guinea pigs, however, could be shown to harbour latent virus in their nervous system.

The surprising finding that HSV could be recovered preferentially from the skin of the footpad during the clinically quiescent phase of the infection offers two alternative interpretations. First, in contrast to the well documented restriction of latent HSV infections to tissues of the nervous system in mice and rabbits, guinea pigs may harbour latent HSV type 2 preferentially and latent HSV type 1 exclusively in the skin at the site of the primary inoculation. Alternatively, HSV infections of guinea pigs may be no exceptions to the rule that latent virus resides exclusively in nervous tissues. Such latent virus may, however, become frequently or even consistently reactivated in the ganglia to spread via peripheral nerves into the skin, although overt clinical disease may be only rarely induced. The latently infected ganglia may harbour only small amounts of virus, frequently escaping detection in our assay, whereas the skin, and in some animals also the nerve trunks, may contain enough reactivated virus to be more easily recovered.

The second explanation seems more likely as it is essentially consistent with data, obtained in other species. In addition, it could support a hypothesis on the pathogenesis of recurrent herpes infections, suggested recently by Hill and Blyth¹⁴. According to this theory, virus is frequently activated in ganglia, travels to the skin, and induces micro-foci of infected epidermal cells, which are usually eliminated by the host's defence mechanisms. Visible lesions develop only under some additional alteration of the local environment. If this theory is correct, virus should be frequently detectable in the skin. The previous failures to recover virus from skin of mice and men^{9,10} using organ culture may be due to the comparatively rare spontaneous reactivation of the latent virus within the ganglia in these species.

Further experiments in neurectomised guinea pigs should help to determine which of these alternative interpretations is correct. Evidence was given in a previous report¹², that HSV travels along the sciatic nerve from skin to ganglia and vice versa. Dissection of the nerve before infections should therefore prevent the establishment of latent infection in ganglia. Consequently, in such animals neither

Table 2 Recovery of virus from animals infected with HSV type 1

Day after infection	Virus recovered (animals positive/animals tested) from			
	Footpad	Sciatic nerve	Dorsal root ganglia (L ₄ -S ₃)	Lumbosacral spinal cord
79	0/2	0/2	0/2	0/2
198	2/2	0/2	0/2	0/2
313	4/4	0/4	0/4	0/4

recurrent lesions nor latent virus in the skin of the footpad should be found, if the second explanation holds. Such experiments are currently under way.

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Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture

WHEN chondrocytes from sternal or articular cartilage are kept in monolayer culture at low density, they eventually lose their cartilage phenotype^{1–4}. Within four passages or approximately 1 month in culture they change from a polygonal or round to a flattened, amoeboid-like shape^{5–7}, and instead of cartilage collagen (type II collagen⁸) they synthesise the genetically different type I collagen. It is not known whether there is a strict correlation between the occurrence of cell flattening and the change in collagen synthesis within individual cells. We have reported that preferentially flattened, fibroblast-like cells at the edge of cartilage colonies synthesise type I collagen, whereas round or polygonal chondrocytes generally synthesise type II collagen^{1–3}. The change is nearly complete in a culture at a time when excessive flattening is observed⁴. Using an immunofluorescence double staining technique^{9,10}, we have now found that there is no strict correlation between cell morphology and type of collagen synthesised.

We used chondrocytes from 16-day embryonic chick sternal cartilage, which were released by treatment with trypsin and collagenase^{1–3} and cultured on tissue culture plastic dishes at a density of 10^4 cells per cm^2 in Ham's F12 medium and 10% foetal calf serum. In these conditions the change from type II to type I collagen synthesis was nearly complete within 1 month.

The type of collagen synthesised by individual cells was determined at various stages of dedifferentiation by immunofluorescence using specific antibodies to type I and type II collagen⁹. This facilitated the comparison of cell shape and the type of collagen synthesised. The double staining procedure enabled us to determine whether a single cell could synthesise both types of collagen simultaneously¹⁰. During the first day of culture the chondrocytes remained almost round; staining with rabbit anti-type II collagen antibodies revealed strong fluorescence of the cell membrane and of numerous filopodia (Fig. 1a). Because of strong membrane-bound fluorescence no intracellular type II collagen or procollagen could be detected. No positive reaction with antibodies to type I collagen was obtained at this stage. After 2 d most chondrocytes had settled on the surface, flattened and retracted their filopodia. These cells showed little membrane bound fluorescence, for the nucleus appeared dark after staining with anti-type II collagen antibodies, but strong intracellular fluorescence (Fig. 1b). This was probably due to type II procollagen, the intracellular form of collagen¹¹, which seemed to be packed in

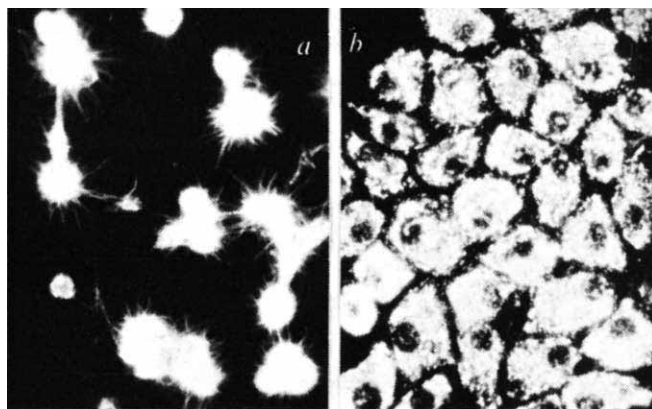


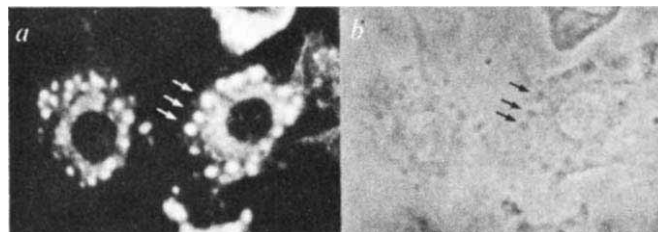
Fig. 1 Immunofluorescent staining of chondrocytes from 16-day embryonic chick sterna with antibodies against type II collagen after 24 h in culture. Chondrocytes were released from sterna by treatment with 0.25% trypsin and 0.1% crude collagenase for 90 min at 37 °C, washed with F12 medium plus 10% foetal calf serum, and plated on Falcon plastic dishes No. 3001 at cell densities of 10^4 cells per cm^2 in Ham's F12 medium and 10% foetal calf serum^{1–3}. After 24 h most cells had attached to the surface, but none were flattened; they showed strong membrane-bound fluorescence and numerous filopodia. For immunofluorescent staining, cells were washed with saline and fixed with 70% ethanol followed by 98% ethanol-ether (1:1, v/v). The air dried cells were reacted with monospecific rabbit antibodies to chick type II collagen (0.12 mg ml^{-1}) for 30 min at room temperature, washed and counterstained with fluorescein conjugated goat anti-rabbit γ globulin as described earlier⁹. Preparation and specificity of the antibodies was described previously⁹. Cells did not stain with antibodies to type I collagen at this time in culture. $\times 260$. **b**, Immunofluorescent staining (see Fig. 1) of a chondrocyte colony after 48 hours in culture. Cells have flattened and retracted their filopodia. Fluorescence with antibodies to type II collagen is located mostly intracellularly as indicated by the weak staining of the membrane above the nuclear area. $\times 260$.

large vacuoles in some cells (Fig. 2). These vacuoles appeared dark in the phase contrast microscope after fixation and antibody staining (Fig. 2b) but were not visible in living cells. It cannot be ruled out that they were an artefact of the fixation and drying procedure, but we assume that they are secretory granules filled with procollagen¹².

After 2 d in culture the first cells started to produce type I collagen. Biochemical determinations have shown that after 2 weeks about 50% of the newly synthesised collagen was type I collagen^{1–3}. Double staining with antibodies to type I and type II collagen revealed that most cells synthesised either type I or type II collagen (Fig. 3). Only in about 1 out of 100 cells was fluorescence observed with both antibodies. There was no significant difference in the morphologic appearance of type I and type II collagen synthesising cells. We observed cells of nearly identical shape in close proximity, which may have originated from the same mother cell, one synthesising type I and the other type II collagen (Fig. 3, arrows).

Type I as well as type II collagen synthesising binucleated cells were observed in increasing amount with prolonged

Fig. 2 Immunofluorescence (a) and phase contrast micrograph (b) of the same cells of a 12-day chondrocyte culture after fixation and staining with antibodies to type II collagen. Fluorescence seems to focus in large intracellular vacuoles (arrows) which may be secretory granules containing type II procollagen. The phase contrast is low because the cells were fixed and air dried. This kind of vacuole was not seen in phase contrast microscopy of living cells. ($\times 478.5$).



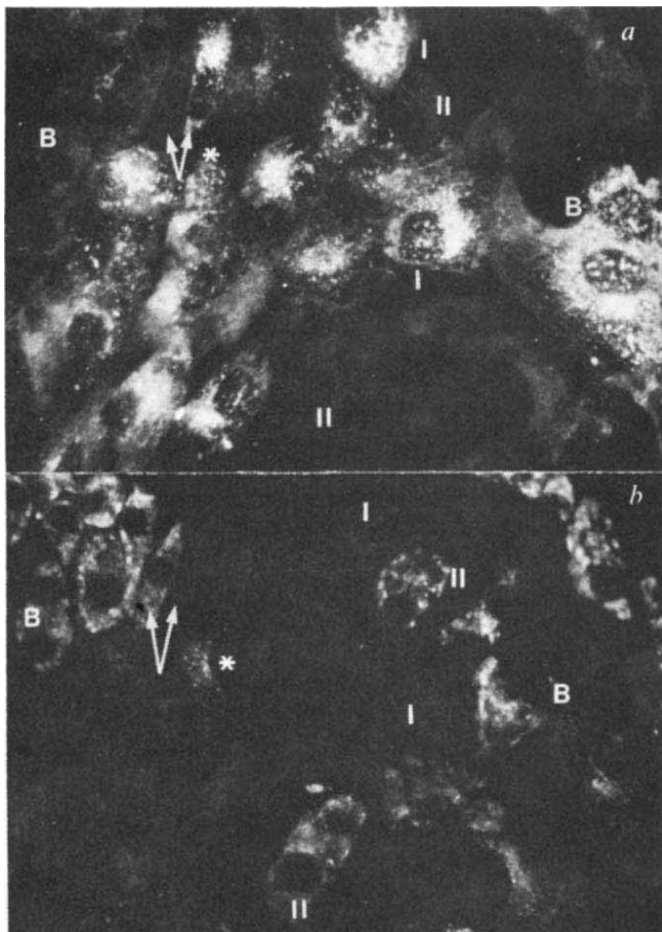


Fig. 3 Immunofluorescent double staining of a chondrocyte culture after 14 d in culture with rabbit antibodies to type I collagen followed by rhodamin conjugated goat anti-rabbit γ -globulin, and guinea pig antibodies to type II collagen followed by fluorescein conjugated rabbit anti-guinea pig γ -globulin⁹. In (a) the fixed culture was photographed with a red filter Rf1 580 (Zeiss), revealing only type I collagen-bound rhodamine fluorescence, and (b) shows type II collagen-bound fluoresceine reaction of the same cell group, photographed through a KP490 filter (Zeiss)⁹. Most cells synthesise either type I (I) or type II (II) collagen except for the cell labelled by an asterisk (*) which shows fluorescence with both types of antibodies. Type I and type II collagen synthesising cells are often located in close proximity and of nearly identical cell shape (arrows). Binucleated cells (B) may produce type I or type II collagen. $\times 360$.

time in culture (B in Fig. 3), which may have been created by incomplete mitosis or cell fusion.

This experiment demonstrates, first, that no conclusions can be drawn from the morphological appearance of a cultured chondrocyte about the type of collagen the cell is producing. Second, because only a few cells were observed which synthesised type I and type II collagen simultaneously, it is evident that the cells do not change gradually from type II to type I collagen synthesis, but rather abruptly. Since the overall transition from type II to type I collagen synthesis in a culture is a gradual process extending through several weeks¹⁻⁴, individual cells must 'switch' at different times in culture. This is consistent with the observation of an increasing number of type I collagen producing cells during the cell culture period, associated with a decreasing number of type II collagen producing cells.

The reason for individual differences in the stability of the chondrocyte phenotype within the cell population obtained after dissociation of embryonic sterna is still unclear. It is possible that young chondroblasts derived from regions underneath the perichondrium had to undergo more doublings to dedifferentiate than did older chondrocytes from the centre of the sternum which were closer to hypertrophy than subperichondral cells. We have also

observed that in general cells located at the periphery of cartilage making colonies³ or cells in between those had changed to type I collagen synthesis earlier than cells from the centre of the colony. This indicates that the stability of the chondrocyte phenotype is also dependent on cell-cell or cell-matrix interactions.

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Effect of prostaglandin endoperoxides and metabolites on bone resorption *in vitro*

PROSTAGLANDINS are potent bone resorbers¹ which have been implicated as mediators of bone loss and hypercalcaemia in disease²⁻⁶, but the specific products in the pathway of prostaglandin synthesis and degradation which are responsible have not yet been identified *in vivo*. Although PGE₂ is the most potent stimulator of bone resorption among the prostaglandins thus far tested *in vitro*⁷, in animal tumour models, the increase in immunoreactive PGE₂ concentrations does not correlate well with the development of hypercalcaemia². We therefore tested the early products of arachidonic acid metabolism via the cyclo-oxygenase pathway, the prostaglandin endoperoxides (PGG₂ and PGH₂) and some of the 13,14-dihydro (H-PGE₂ and H₂-PGE₁) and 15-keto-13,14-dihydro metabolites of prostaglandins (15K-H₂-PGE₂ and 15K-H-PGF_{2α}) for their effects on the release of previously incorporated ⁴⁵Ca from cultured foetal rat long bones. The endoperoxides were found to cause a rapid transient increase in the release of previously incorporated ⁴⁵Ca from bone, but did not stimulate prolonged resorption. H₂-PGE₂ and H₂-PGE₁ were almost as potent as the parent compounds. 15K-H₂-PGE₂ and 15K-H-PGF_{2α} were much less potent, but did stimulate resorption at 10⁻⁶ M.

The diseases in which increased prostaglandin production has been implicated as the mediator of pathologic bone loss include malignancy^{2,3}, rheumatoid arthritis⁴, and periodontal disease^{5,6}. Bone itself can produce prostaglandin when cultured with antibodies to cell surface components and complement⁸. Moreover, PGE₂ can inhibit bone collagen synthesis as well as stimulate bone resorption⁹.

There is clear evidence for an increased metabolism of arachidonic acid via the cyclo-oxygenase pathway in the hypercalcaemia of malignancy, as markedly increased levels of PGE₂ metabolites have been found in the blood of hypercalcaemic mice with the HSDM₁ fibrosarcoma¹⁰ and hypercalcaemic rabbits bearing the VX₂ tumour¹¹ and in the urine of patients with the hypercalcaemia of cancer². It has been more difficult, however, to prove that PGE₂ itself is the circulating material responsible for hypercalcaemia. In animal tumour models a modest increase in immunoreactive PGE₂ is seen only after hypercalcaemia has developed², and in patients

with the hypercalcaemia of cancer the evidence for increased circulating immunoreactive PGE₂ is conflicting^{12,13}. Low levels of circulating PGE₂ in the presence of its increased synthesis are most likely the result of its rapid metabolism by the lung and other tissues so that PGE₂ itself may not accumulate in the circulation¹⁴. This raises the possibility that either metabolites of PGE₂ or other products of arachidonate metabolism via the cyclo-oxygenase pathway such as thromboxane or prostacyclin¹⁵ might stimulate osteoclastic activity and produce hypercalcaemia.

Accordingly, we have evaluated the bone resorption produced by PGG₂ and PGH₂, the labile cyclic endoperoxides from which all prostaglandins and thromboxanes are formed¹⁶. We have also examined the major metabolite of PGE₂ that persists in the circulation, 15-keto-13,14-dihydro-PGE₂ (15K-H₂-PGE₂), the major product of metabolism by the lung, as well as 13,14-dihydro-PGE₂ (H₂-PGE₂) which is produced by reduction of the 15-keto group of 15K-H₂-PGE₂ in organs such as the liver¹⁷. Finally, we compared some analogous compounds, PGE₁, H₂-PGE₁ and 15K-H₂-PGF_{2α}.

The endoperoxides, PGG₂ and PGH₂, were prepared according to the method of Hamberg *et al.*¹⁸; following isolation by silicic acid chromatography their purity and structures were confirmed by thin layer chromatography and mass spectrometry of their respective reduction and isomerisation products¹⁹. H₂-PGE₂, H₂-PGE₁, 15K-H₂-PGE₂ and 15K-H₂-PGF_{2α} were prepared by incubation of ³H-labelled and unlabelled precursors with homogenates of guinea pig liver by the method of Hamberg and Israelsson¹⁷. The products were resolved initially by reversed phase partition chromatography with solvent system C-50 (ref. 20) and further purified with high performance liquid chromatography²¹. The structures were confirmed by mass spectrometry. PGE₂ and its metabolites were quantified on the basis of radioactivity after standardisation by gas chromatography.

The effects of prostaglandin endoperoxide and metabolites on bone resorption were assessed by measuring the release of previously incorporated ⁴⁵Ca from foetal long bones cultured in a chemically defined medium with 4 mg ml⁻¹ bovine serum albumin as described previously^{7,22}. The bones were precultured for 18–24 h to remove exchangeable ⁴⁵Ca. The endoperoxides were stored at –80 °C in acetone and were injected into the culture medium in 1 µl of acetone per 500 µl of medium. Controls received acetone alone. The metabolites were added in alcohol (less than 0.1% final concentration). Controls received alcohol alone. ⁴⁵Ca release was measured in medium and bone and the per cent of total bone ⁴⁵Ca released during each culture period calculated.

The prostaglandin endoperoxides, PGG₂ and PGH₂, did not produce consistent stimulation of bone resorption in 48-h cultures when injected at 3–12 h intervals. Their effect was always less than that of equivalent amounts of PGE₂ administered in an identical manner. Because of their short half life, the effects of more frequent injections were examined. Bone cultures were injected hourly with endoperoxides or PGE₂ for 5 h and calcium release measured at 6 h. The cultures were then transferred to fresh medium to see if further resorptive responses occurred. Using this schedule, PGG₂ at 10⁻⁶ and 10⁻⁸ M and PGH₂ at 10⁻⁶ and 10⁻⁷ M increased the release of ⁴⁵Ca from foetal bones at 6 h (Table 1). The effect of PGH₂ at 10⁻⁸ M was tested in four additional experiments; in two of these ⁴⁵Ca release was increased significantly by 20–25%. Hence 10⁻⁸ M PGH₂ is apparently at or near the threshold concentration for early ⁴⁵Ca release. PGE₂ at 10⁻⁶ to 10⁻⁸ M did not produce a significant 6-h increase in ⁴⁵Ca release in any experiment. After 6 h of treatment with PGE₂, however, there was always a significant resorptive response during the subsequent 42 h of culture. The 6–48 h response after endoperoxide treatment was quite variable and usually not statistically significant. A prolonged response to brief exposure has been observed with other stimulators of bone resorption²³ and has been termed induction. Because the rapid effects of

Table 1 Effect of PGG₂, PGH₂ and PGE₂ on release of previously incorporated ⁴⁵Ca from cultured foetal rat long bones

Treatment	% of total bone ⁴⁵ Ca release 0–6 h	0–48 h
Experiment 1		
Control	8.2±0.4	21.7±0.6
PGG ₂ 10 ⁻⁶ M × 5	10.7±0.5*	27.5±3.2
10 ⁻⁸ M × 5	10.4±0.7*	24.4±1.5
PGH ₂ 10 ⁻⁶ M × 5	12.2±0.7*	30.7±4.2
10 ⁻⁸ M × 5	9.2±0.3	20.6±0.5
PGE ₂ 10 ⁻⁶ M × 5	8.4±0.3	30.2±2.4*
10 ⁻⁸ M × 5	8.5±0.5	28.0±1.8*
Experiment 2		
Control	8.5±0.4	27.8±2.7
PGH ₂ 10 ⁻⁷ M × 5	15.8±1.2*	29.2±1.9
PGE ₂ 10 ⁻⁶ M × 5	9.2±0.2	45.3±1.8*

Values are means ± s.e. for four to twelve bone cultures treated with 5-hourly injections of prostaglandin in acetone beginning at 0 time; controls received acetone alone. Cultures were transferred to fresh medium without prostaglandins or acetone at 6 h.

*Significantly different from controls, *P* < 0.01.

endoperoxides were small in terms of total bone calcium, it was not possible to determine whether there was stimulation of matrix resorption as well as calcium release at 6 h. The initial effect could have been due to an increase in cell permeability to calcium²⁴. High calcium concentrations in the medium have previously been shown to inhibit induction by parathyroid hormone²⁰. It is possible that the calcium released by endoperoxides could decrease the subsequent resorptive response even if a substantial amount of PGE₂ were produced. It is also possible that the endoperoxides produced tissue damage which impaired resorptive response. We have, however, observed that endoperoxide-treated bone can respond to subsequent addition of both PGE₂ and parathyroid hormone (data not shown).

The metabolites of PGE₂, PGE₁ and PGF_{2α} which may have a longer half life in the circulation than the parent compounds were tested only in prolonged continuous cultures. Both H₂-PGE₂ and H₂-PGE₁ were nearly as potent as the parent compounds (Fig. 1) and as previously reported⁸ PGE₁ was somewhat less potent than PGE₂. In contrast, 15K-H₂-PGE₂ and 15K-H₂-PGF_{2α} were much less effective. Some stimulation of resorption was observed at 10⁻⁸ M (Table 2). In another experiment 89% of total ⁴⁵Ca released after 5 d treatment with 15K-H₂-PGE₂ at 2 × 10⁻⁵ M, but at 2 × 10⁻⁴ M, the highest concentration tested, 15K-H₂-PGE₂ actually inhibited ⁴⁵Ca

Fig. 1 Effect of prostaglandins E₂ (●) and E₁ (■) and their 13,14-dihydro metabolites, H₂-PGE₂ (○) and H₂-PGE₁ (□), on release of previously incorporated ⁴⁵Ca from cultured foetal rat long bones. Data from eight experiments were pooled and the points are means for 6–22 cultures incubated for 5 d with medium changed at 2 d. Standard errors were 5 to 15% of the means. The differences from control at 10⁻⁷ and 10⁻⁶ M were significant at *P* < 0.01, at 10⁻⁸ M at *P* < 0.05. Broken line represents control levels. *a*, Release at 2 d; *b*, release at 5 d.

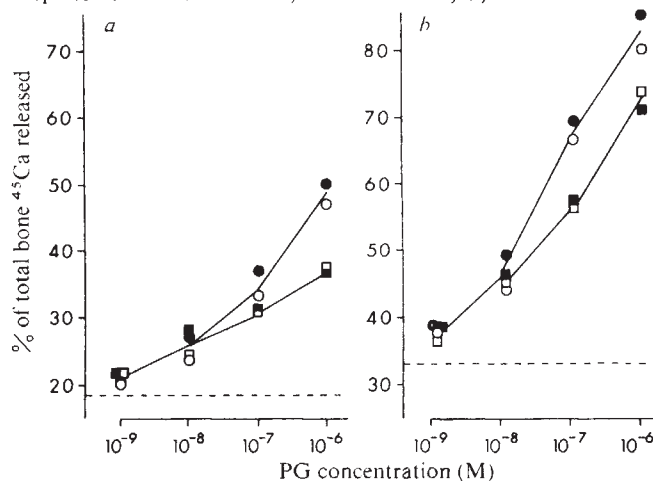


Table 2 Effect of 15K-H₂-PGE₂ and 15K-H₂-PGF_{2α} on release of previously incorporated ⁴⁵Ca from cultured foetal rat long bones

Treatment	% of total bone ⁴⁵ Ca released
Control	40.3±4.1
15K-H ₂ -PGE ₂ 10 ⁻⁷ M	43.8±5.0
10 ⁻⁶ M	48.0±6.4
10 ⁻⁵ M	54.1±2.9*
15K-H ₂ -PGF _{2α} 10 ⁻⁷ M	38.0±4.3
10 ⁻⁶ M	38.2±3.7
10 ⁻⁵ M	55.5±6.4*

Values are mean±s.e.m. for four to eight bones cultured for 5 d. Medium was changed at 2 d.

*Significantly different from controls, $P < 0.05$.

release to 25% compared with a control of 41%. The inhibition may be a toxic effect and has been seen with other prostaglandins at high concentrations⁸. At their maximally effective doses, all the agents tested produced similar morphological changes with almost complete resorption of mineral and matrix at 5 d. To exclude any possible contribution of different binding to albumin to the relative potencies of these compounds, a separate experiment was performed in which medium without albumin was used, and PGE₂ stimulated bone resorption significantly at 10⁻⁸ M while 15K-H₂-PGE₂ was again ineffective at 10⁻⁶ M. These results indicate that the 13,14-dihydro derivatives are nearly as potent stimulators of bone resorption as PGE₂ and PGE₁, whereas 15K-H₂-PGE₂ is considerably less potent. Atkins and Martin²⁵ found a similar relation between metabolites and parent compounds for stimulation of cyclic AMP production by osteogenic sarcoma cells. It will be of interest to determine whether any of these compounds can accumulate sufficiently in the circulation to contribute to the resorption of bone in the hypercalcaemia of cancer.

The prostaglandin endoperoxides, PGG₂ and PGH₂, cause a transient stimulation of calcium release that is quite different from the prolonged resorption resulting from PGE₂. These findings raise the possibility that bone tissue enzymically converts PGG₂ and PGH₂ to a labile product such as thromboxane A₂ or prostacyclin (PGX). Further studies will be required to delineate which of the products of arachidonate metabolism via the cyclooxygenase pathway account for the resorption of bone in neoplasia and inflammation.

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Increased cyclic GMP levels associated with contraction in muscle fibres of the giant barnacle

CYCLIC GMP has been implicated as an intracellular second messenger in a variety of systems. In particular, a number of hormones and neurotransmitters have been shown to raise cellular cyclic GMP levels *in vitro*^{1–9}. In many of these systems, however, Ca²⁺ entry seems to be responsible both for the physiological effect of the hormone and for the hormone-stimulated increase in cyclic GMP (refs 4–9). We have investigated the relationship between calcium and cyclic GMP levels in muscles of the giant barnacle *Balanus nubilus*. This preparation has a number of advantages. Within fairly narrow limits intracellular free calcium levels in the resting muscle are known¹⁰. The depolarisation-induced increase in free calcium, and the resulting activation of contraction, have also been extensively documented^{10,11}. In addition, the depolarisation-induced changes in intracellular free calcium can be easily manipulated because the extracellular fluid is the apparent source of the calcium which couples excitation to contraction¹². We report here that either KCl depolarisation or nerve stimulation increases the cyclic GMP content of barnacle muscle. This increase seems to be associated with Ca²⁺ entry rather than with a neurotransmitter–receptor interaction *per se*. This is the first report of an increase in cyclic GMP associated with contractile activity in cross-striated muscle.

The three pairs of depressor muscles were used in all the experiments described below. The dissection of these muscles and their nervous supply has been described previously¹³. The cyclic GMP content of resting barnacle muscle, measured according to the procedure described in Fig. 1, was 75±8 fmol per mg protein (mean±s.e.m. for 34 individual resting muscles from a total of 18 barnacles).

Depolarisation of the muscles by elevating the KCl concentration of the barnacle saline for 2 min produced an increase both in isometric tension and in cellular cyclic GMP (Fig. 1). The isometric tension increased rapidly and reached a near-maximal value within 15 s. The cyclic GMP content was measurably elevated within 30 s and continued to increase until 2 min, at which time it was nearly three times control. Beyond this time, the cyclic GMP level fell towards that of resting muscle, even when the exposure to KCl was maintained. The cyclic GMP content declined toward the basal level despite the fact that the muscle was able to maintain a constant isometric tension for as long as the KCl stimulus was present (longest time tested=5 min, data not shown). These results indicate that the cyclic GMP level does not directly control the contractile state. (Although we have not established the reason for the decline in cyclic GMP, it may be physiologically important; for instance, Ca²⁺ entry or some other event associated with muscle contraction might cause a rapid increase in the synthesis, and a slower increase in the degradation, of the nucleotide.) If, after 2 min of KCl depolarisation, the muscles were returned to normal barnacle saline, isometric tension decreased to the pre-stimulus level and the fall in cyclic

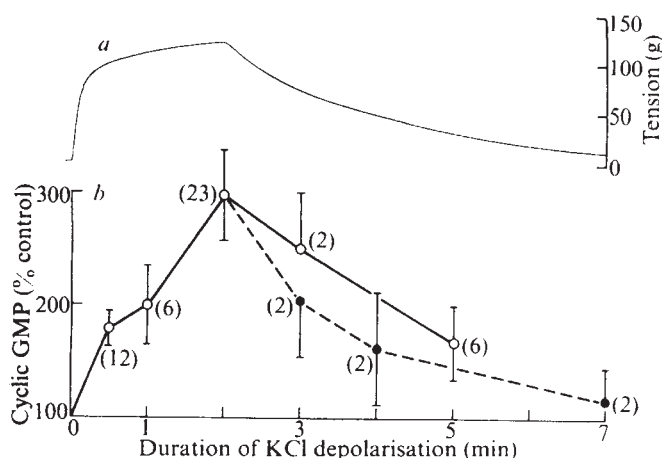


Fig. 1 Effect of KCl depolarisation on isometric tension (a) and cyclic GMP content (b) of giant barnacle muscle. The tension trace shows the effect of a 2-min exposure of the muscle to 100 mM KCl-barnacle saline followed by a return to control barnacle saline. The control barnacle saline (modified from ref. 13) had composition (mM): Na^+ , 476; K^+ , 8; Ca^{2+} , 20; Mg^{2+} , 12; Cl^- , 548; MOPS (3-(N-morpholino)propanesulphonic acid), 10; pH adjusted to 7.4 with NaOH. The 100 mM KCl-barnacle saline was made by equimolar substitution of K^+ for Na^+ in the control saline. The cyclic GMP data were calculated as follows: from each barnacle 1–5 muscles were used as test muscles and 1–3 muscles as control muscles. The cyclic GMP content of an individual test muscle was expressed as $100 \times (\text{pmol cyclic GMP per mg protein of the test muscle} \div \text{mean pmol cyclic GMP per mg protein of control muscle from the same barnacle})$. Thus, each barnacle yielded 1–5 experimental values. ○, Mean percentage ($\pm$ s.e.m.) cyclic GMP content, relative to control as described, for test muscles incubated for the indicated duration in 100 mM KCl-barnacle saline; ●, mean percentage cyclic GMP content, relative to control, of muscles returned to normal barnacle saline after 2 min in 100 mM KCl-barnacle saline. In all cases, control muscles were incubated in normal barnacle saline for a time equivalent in duration to the incubation of experimental muscles. All muscles were dissected and pre-incubated (1–3 h at 4 °C) in control barnacle saline. Experiments were carried out at room temperature (19–21 °C). After experimental incubations, cyclic nucleotides were extracted by rapidly homogenising the muscles with an Ultraturrax in 7–10 ml ice-cold 7% TCA. Alternatively, the muscles were first frozen in liquid nitrogen and then homogenised in TCA. The cyclic GMP level of resting muscle did not depend on which of these two treatments was used. The TCA-precipitated proteins were pelleted by centrifugation; after removing the supernatant the protein content of the pellets was measured by the method of Lowry *et al.*²². Aliquots (3 ml) of the supernatant were extracted five times with 10 ml water-saturated ether, lyophilised and reconstituted with 0.5 ml 50 mM sodium acetate, pH 6.2. Cyclic GMP was determined by the method of Steiner *et al.*²³ and cyclic AMP by the method of Brown *et al.*²⁴. The recovery of exogenous cyclic GMP and cyclic AMP, added to whole muscle homogenates, was 85% and 90%, respectively. Treatment of the sodium acetate-reconstituted samples with beef heart phosphodiesterase (Boehringer) reduced their cyclic nucleotide content essentially to zero.

GMP seemed to be slightly more rapid than in the continued presence of high KCl although the difference was probably not statistically significant (Fig. 1).

KCl depolarisation seemed to have relatively little effect on the cyclic AMP content of barnacle muscle, but these data are less complete. The basal cyclic AMP level was 3.57 ± 0.41 pmol per mg protein (mean $\pm$ s.e.m.; 12 muscles from 7 barnacles). Depolarisations ranging from 30 s to 2 min caused an increase of $19 \pm 6\%$ above control (14 muscles from 7 barnacles).

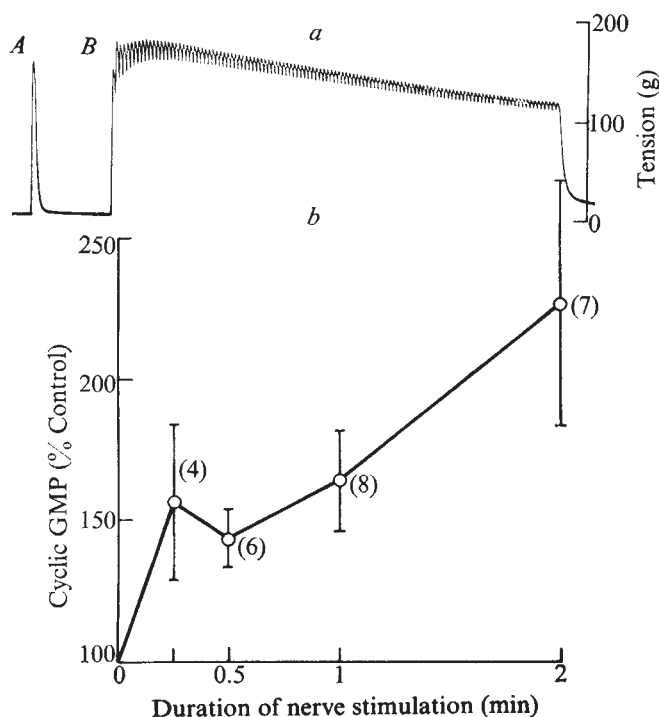
The effect of nerve-evoked contraction on the intracellular level of cyclic GMP is shown in Fig. 2. Repetitive nerve stimulation caused a maintained increase in tension, and a time-dependent increase in muscle cyclic GMP, during the interval studied.

To determine whether, as in many other systems^{4–9}, the stimulated increases in barnacle muscle cyclic GMP level are

dependent on extracellular Ca^{2+} , muscles were soaked for 2–3 h in Ca^{2+} -free barnacle saline. After such treatment, depolarisation with Ca^{2+} -free 100 mM KCl-barnacle saline caused no visible contraction and no increase in cyclic GMP (Table 1, B1). The absence of an increase in cyclic GMP was not a result of deterioration of the muscle. Exposing muscles which had been preincubated in Ca^{2+} -free barnacle saline to Ca^{2+} -containing 100 mM KCl-barnacle saline caused a strong contraction and an increase in cyclic GMP comparable to that for muscles which had been preincubated in normal barnacle saline (compare B2 with A2 in Table 1).

Based on studies of other crustaceans¹⁴, glutamate is probably the excitatory neuromuscular transmitter in the barnacle. As support for this contention we found that bath applied glutamate (0.1–1.0 mM) caused barnacle muscle to contract; but this contraction was weak and variable, with a duration of usually less than 10 s. In such conditions (0.5 mM glutamate for 2 min), there was no alteration in muscle cyclic GMP ($101 \pm 18\%$, relative to control, for six muscles). The bath application of higher (10 mM) concentrations of glutamate caused a vigorous, brief (<1 s) contraction. The brevity of this contraction is probably a consequence of the rapid desensitisation of the muscle glutamate receptors. This desensitisation to applied glutamate was accompanied by a rapid reduction in the visible contraction induced by nerve stimulation, further supporting the hypothesis that glutamate is the excitatory neurotransmitter. Complete abolition of the response to nerve stimulation required an exposure to 10 mM glutamate

Fig. 2 Effect of nerve stimulation of varying duration on isometric tension (a) and on cyclic GMP content (b) of giant barnacle muscle. The time scale applies to parts A and B of the tension record as well as to the cyclic GMP data. a, A, Isometric tension response to a single stimulus train applied to the motor nerve. An individual train consisted of 10 pulses delivered at 100 Hz; each pulse was supramaximal in amplitude and 1 ms in duration. a, B, Isometric tension response when such a stimulus train was applied repetitively (once per second for 2 min). b, Each point shows the mean percentage ($\pm$ s.e.m.) of cyclic GMP in stimulated muscles compared with control muscles (calculated as described in Fig. 1). Stimulus conditions were identical to those for a, B. After stimulating the nerve for the indicated periods of time, the muscles (both stimulated and control) were homogenised and further processed as described in Fig. 1.



for 1 h. (This slow time course does not seem unreasonable since many of the individual fibres of the muscle are not directly exposed to the bathing solution and since many of the neuromuscular junctions of individual fibres lie at the bottom of deep, narrow clefts¹⁵.) After neuromuscular transmission was blocked with 10 mM glutamate, stimulation of the motor nerve no longer caused an increase in muscle cyclic GMP level (Table 1, C1). This observation suggests that the nerve-induced (Table 1, A1) increase in cyclic GMP requires the activation of glutamate receptors by the neurotransmitter. KCl depolarisation, however, was still able to increase the cyclic GMP level in glutamate-desensitised muscles (Table 1, C2). This finding indicates that the KCl-induced increase in cyclic GMP is not a result of depolarisation-dependent release of glutamate from the nerve terminals. The simplest hypothesis that could account for the increase in cellular cyclic GMP produced by nerve stimulation and KCl depolarisation, as well as for the fact that the KCl-induced increase required Ca^{2+} , is that both types of contractile stimuli increase cyclic GMP by causing Ca^{2+} influx across the muscle membrane.

In the barnacle, as well as in a number of other systems, receptor activation by the appropriate hormone or neurotransmitter is associated with increased cyclic GMP and a physiological response. In some systems, cyclic GMP seems to serve as a causal link between receptor activation and the physiological response. For example, muscarinic cholinergic agonists increase cyclic GMP levels in the heart¹ and in sympathetic ganglia¹, and exogenous derivatives of cyclic GMP are able to mimic some of the physiological effects of muscarinic agonists in these preparations^{16,17}. In certain other systems in which hormonal elevation of cyclic GMP has been observed, Ca^{2+} seems, however, to link stimulus to response^{3,4,6-8}. It seems likely that the barnacle muscle is one of these latter systems and that calcium rather than cyclic GMP is the agent which directly activates the contractile machinery.

Table 1 Effect of nerve stimulation and of KCl depolarisation on cyclic GMP in giant barnacle muscle in various conditions

Pre-incubation	Stimulation	% Control
A, Control saline	1. Nerve stimulation	227 ± 44 (7)
	2. 100 mM KCl-saline with Ca^{2+}	298 ± 39 (23)
B, Ca^{2+} -free saline	1. 100 mM KCl without Ca^{2+}	99 ± 5 (8)
	2. 100 mM KCl with Ca^{2+}	370 ± 49 (11)
C, Glutamate saline	1. Nerve stimulation	119 ± 13 (5)
	2. 100 mM KCl-glutamate-saline	267 ± 60 (18)

A, Muscles were dissected, and pre-incubated for 1–3 h, in control barnacle saline, as described in Fig. 1. Control muscles were then incubated for an additional 2 min in control barnacle saline; stimulated muscles were incubated for 2 min in 100 mM KCl-barnacle saline or were stimulated through their motor nerve as described in Fig. 2.

B, Muscles were dissected and washed with Ca^{2+} -free barnacle saline (equimolar replacement of the Ca^{2+} in the control saline with Mg^{2+}) and incubated for 2–3 h at 4 °C, with two changes of fresh Ca^{2+} -free saline. Control muscles were then incubated for an additional 2 min in Ca^{2+} -free saline; stimulated muscles were incubated for 2 min in Ca^{2+} -free 100 mM KCl-saline or for 2 min in Ca^{2+} containing 100 mM KCl-saline.

C, All muscles were pre-incubated for 1–3 h at 4 °C in control barnacle saline, and then incubated for an additional hour at 20 °C in barnacle saline plus 10 mM monosodium glutamate ('glutamate saline'). Control muscles were then incubated for an additional 2 min in glutamate saline. Stimulated muscles were either maintained for 2 min in glutamate saline, during which time the motor nerve was stimulated as described in Fig. 2, or else were incubated for 2 min in 100 mM KCl-saline containing 10 mM monosodium glutamate.

Data: mean value ± s.e.m. (number of individual experimental muscles).

Control levels of cyclic GMP were: A, 75 ± 8 ($n = 34$); B, 43 ± 4 ($n = 16$); C, 62 ± 6 ($n = 18$) fmol per mg protein. Note that the basal cyclic GMP level in muscles pre-incubated in Ca^{2+} -free barnacle saline (B) was only about half that of muscles pre-incubated in normal saline (A).

Since Ca^{2+} entry seems to be responsible both for the activation of contraction in the barnacle, and for the increase in cyclic GMP, the problem remains as to the function of the cyclic GMP increase associated with contractile activity in the muscle fibres of the barnacle. One possibility^{18,19} is that cyclic GMP is involved in regulating Ca^{2+} metabolism. Ong and Steiner²⁰ have suggested that cyclic GMP may have a role in the regulation of myosin function. Another possibility worth considering is that cyclic GMP has a role in activity-dependent control of the expression of the muscle genome. In mammalian skeletal muscle, for example, muscle activity can prevent the appearance of denervation-induced extrajunctional acetylcholine receptors²¹. In preliminary experiments, we have found that contractile activity in vertebrate skeletal muscle is associated with an increase in cyclic GMP level.

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Development of cholinergic characteristics in adrenergic neurones is age dependent

OBSERVATIONS from several laboratories indicate that neurones from adrenergic ganglia cultured from rats during the perinatal period may, in certain culture conditions, develop several clearly cholinergic characteristics. These cultured neurones may form cholinergic synapses among themselves as well as on cocultured striated muscle^{1–3}, and the cell population developing cholinergic mechanisms derives from an initially adrenergic neuronal population as a gradual shift in synaptic vesicle cytochemistry occurs together with increasing choline acetyltransferase (ChAc) activity and an increasing number of cholinergic synaptic interactions between neurones^{4,5}. In certain conditions these cultured neurones may release both noradrenaline and acetylcholine⁶. We address here the question of whether this unexpected ability of the adrenergic neurone to develop cholinergic mechanisms in culture derives from the neurone's response to some novel aspect of the tissue culture system

or is instead the *in vitro* expression of a developmental period during which the automatic neurone *in vivo* is in a labile state of differentiation. Our results favour the latter explanation.

These experiments were made possible by two developments. First, small explants of superior cervical ganglia (SCG) were successfully cultured from increasingly older

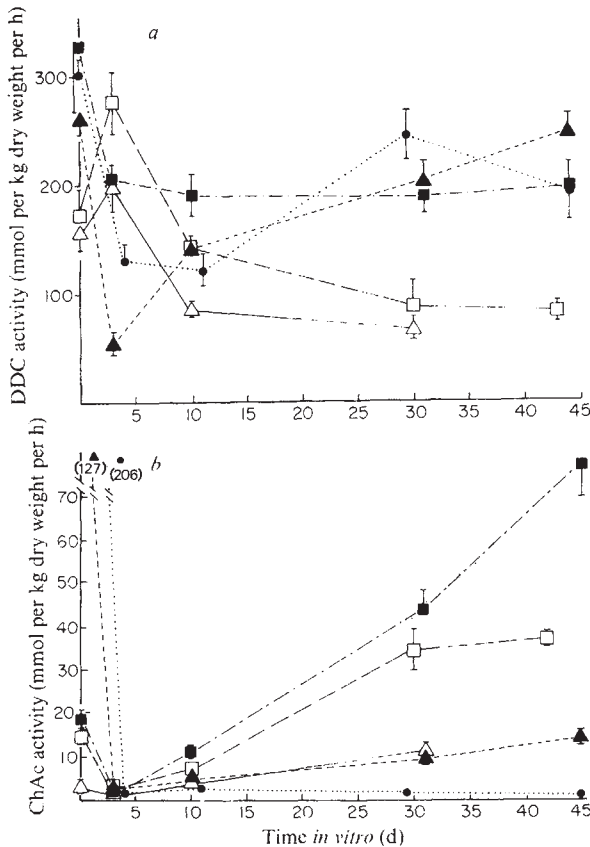


Fig. 1 *a*, Variation of DOPA-decarboxylase (DDC) activity in SCG explants with time in culture. Ganglia were obtained from 15½ (△) and 20 (□) day embryonic and 2 (●), 5 (not shown), 15 (▲), 22 (not shown), and 200 (●) (adult) day postnatal rats. Explants of approximately equal size were obtained by culturing the entire 15½-day embryonic ganglion and by dividing the increasingly larger, more mature ganglia into pieces of similar size. Explants were placed on to 24-mm Aclar dishes and fed three times weekly with medium containing 65% Eagle's minimum essential medium with glutamine added, 25% human placental serum (HPS) 10% chick embryo extract (EE), 3% 1.1 M glucose, and 25 units ml⁻¹ nerve growth factor. These levels of HPS and EE have been shown to foster the development of cholinergic properties in cultures of SCG neurones⁷. At the times indicated, cultures were rinsed with Leibovitz medium and, after carefully blotting excess medium, were frozen and stored at -80 °C. Cultures were freeze-dried under vacuum at -40 °C (ref. 18) for 3 d. Explants were dissected free from supporting cell and neurite outgrowth and from the collagen, weighed using a quartz-fibre balance¹⁸, and homogenised in 36 µl of 50 mM sodium phosphate buffer (pH 7.6) containing 0.1% bovine serum albumin and 0.1% Triton X-100. Intact ganglia for *in vivo* (or zero time) enzyme activities were treated in the same way except that ganglia from older animals were homogenised in larger volumes in order to keep the weight-volume ratio constant. Homogenates were stored at -80 °C until assayed. DDC activity was determined by a modification of the method of McCaman *et al.*¹⁹. Enzyme activity is expressed relative to dry weight of the explant ± s.e.m. There were no significant differences between weights of explants from different age rats after the same time in culture. The DDC activity in explants from postnatal animals after 30 d in culture stabilises at about 70% of the activity in the adult ganglia *in vivo*. Studies of thionin-stained explants are in progress to determine the number of neurones in explants from different age animals after 40 days in culture. *b*, Variation of choline acetyltransferase (ChAc) activity in SCG explants with time in culture. ChAc activity was determined by a modification²⁰ of published methods^{21,22} on the same homogenates described above. Enzyme activity is expressed relative to dry weight of the explant ± s.e.m. Activity at zero time is the *in vivo* activity in the ganglion.

rats, including adult. A similar pattern of ChAc development was seen in SCG from perinatal rats regardless of whether explants or dissociated neurones were studied⁷. Second, we observed that ChAc, the enzyme synthesising acetylcholine and highly localised to cholinergic neurones, is present only in extremely low amounts in all cultures freshly prepared from rat SCG (after degeneration of the pre-ganglionic endings). Thus it was possible to measure ChAc accrual in cultures from adrenergic ganglia prepared at ages between the 15-d embryo and the adult rat. Our results indicate that the ability of SCG explants to develop cholinergic characteristics reaches a maximum at approximately the second postnatal day; beyond the third postnatal week this 'shift' does not occur.

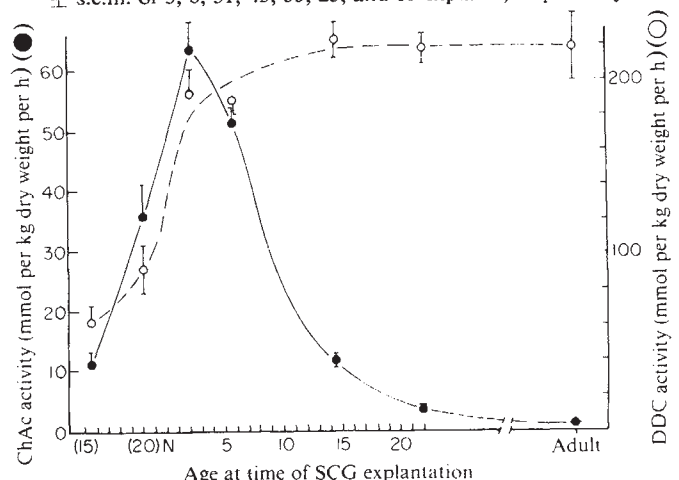
Explants of approximately equal size were prepared from SCG of rats of different ages, cultured in the presence of NGF for 1 month or longer and then assayed for activities of ChAc and of DOPA-decarboxylase (DDC) (details in Fig. 1 legend). The latter enzyme, in the synthetic pathway of catecholamines, is localised to adrenergic neurones in the SCG⁸⁻¹⁰. The cytochemistry of the synaptic vesicle population was studied in cultures from 5-d pups and from adult rats after 1 month in culture by methods previously described¹.

The pattern of change in DDC activity with time in culture was related to whether the explant was taken from a prenatal or a postnatal rat (Fig. 1*a*). DDC activities in SCG explants from 15½-d and 20-d embryos increased during the first 3 d in culture, then decreased during the following 4 weeks. DDC activities in SCG explants from 2, 15, and 22-d (not shown) pups and from adults first decreased, then levelled or increased during the next 4 weeks, beyond which time there were no significant differences between these explants.

The specific activity of ChAc in SCG explants fell during the first 3-4 d in culture (Fig. 1*b*), probably because of degeneration of presynaptic terminals, then increased during the next 4 weeks in all explants except those taken from the 22-d pup (not shown) and the adult animal. The enzyme activity attained by SCG explants from animals of different ages differed significantly after 1 month in culture; the highest activity was in explants from 2-d pups and the lowest activity was in explants from adults.

The variation with rat age of DDC activity in SCG explants after 1 month in culture differed considerably from the variation in ChAc activity in the same explants (Fig. 2). The accrual of DDC activity by SCG explants increased as explants were taken from progressively older

Fig. 2 Activities of DDC and ChAc in SCG explants after 1 month in culture plotted against the age of the rat at the time of explantation. All values for 30 and 45 day points in Fig. 1 for each age were averaged to obtain as large a statistical population as possible. Values for explants from 15½ and 20-d embryos, for 2, 5, 15, and 22 day pups, and for adult animals represent averages ± s.e.m. of 3, 8, 31, 43, 55, 23, and 15 explants, respectively.



animals from the 15½-d embryo to the 2-d pup; explants from animals aged 2 d or older showed no significant differences in the DDC activity after 30 d or more in culture. The ChAc activity after 30 d in culture was higher as explants were taken from progressively older animals from 15½-d prenatal to 2-d postnatal; but the ability of explants from older animals to develop ChAc activity *in vitro* decreased. The ChAc activity in SCG explants cultured from adult animals did not increase at all, the activity after 45 d being significantly less than the activity remaining 4 d after denervation.

In interpreting these data, we must consider the possibility that our culture system selects certain neurones present in the neonatal animal that are already committed to cholinergic. We have presented biochemical, physiological and cytochemical evidence against this view^{4,5}. In the data presented here, we note no significant differences between the ChAc activities in explants from animals of any age 3 to 5 d after denervation (Fig. 1b, and 5-d and 22-d pups not shown), indicating that no cholinergic cells are present at the time of explantation in neonatal SCG that are not present in the adult. The high level of DDC activity in all explants from postnatal animals (Fig. 2) indicates a good state of neuronal viability, and thus the change with rat age (at explantation) in the accrual of ChAc activity *in vitro* must reflect a difference in the neuronal synthesis of ChAc rather than a difference in survival of selected neuronal populations.

The cytochemistry of vesicles within axonal varicosities was consistent with the ChAc data. Vesicles of varicosities in the outgrowth of SCG explants from 5-d pups were

Fig. 3 Electron micrographs of vesicle-containing varicosities found in cultures of SCG explants taken from *a*, 5-d-old rat pups and *b*, adult rats. The cultures were companions to those used for the biochemical analyses (see Fig. 1 legend) and were fixed after 28–33 d *in vitro*. After an initial rinse in Leibovitz medium (L-15) the cultures were incubated 30 min in 10^{-5} M noradrenalin in L-15 to which ascorbic acid (0.2 mg ml^{-1}) was added. The pH was adjusted to 7.2–7.3 before incubation. A rinse in L-15 was followed by fixation with 3% KMnO_4 in Krebs's Ringer phosphate (pH 7.1, 4°C) and by *en bloc* staining with 1% uranyl acetate in maleic acid buffer (pH 5.2, 4°C). Embedding in Epon-Araldite followed dehydration. Scale bar represents 0.3 μm .

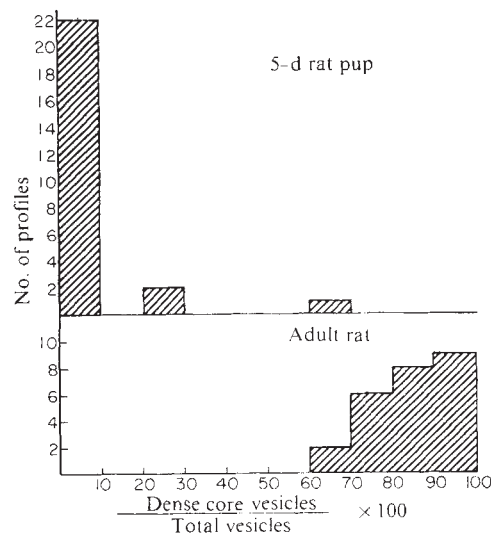


Fig. 4 No. of profiles (ordinate) containing listed percentages of dense-cored vesicles (abscissa) in 28–33-d cultures of SCG from 5-d rat pups and adult rats. Consecutive varicosities, similar to those illustrated in Fig. 3, were photographed and printed at a standard magnification. Vesicles measuring 40–70 nm were counted and classified as dense-cored or clear. Twenty-five profiles from each group are charted. Total vesicles per profile ranged from 9 to 38 and 9 to 54 in the groups taken from 5-d pups and adults respectively.

predominantly clear, whereas the majority of the vesicles of varicosities in explants from adult animals were dense-cored (Fig. 3). This difference is illustrated graphically in Fig. 4. Only 1 of 25 profiles from 5-d pup cultures contained 60–70% dense-cored vesicles and 22 of 25 contained less than 10%. In contrast profiles found in adult rat SCG cultures contained no less than 50% dense-cored vesicles and 17 of 25 contained over 80%.

These data demonstrate that the developmental age of the rat adrenergic neurone determines whether this neurone can adjust its transmitter mechanisms and express in culture two well established characteristics of cholinergic neurones—ChAc activity and clear synaptic vesicles. The dramatic decrease in the ability of SCG explants taken from rats beyond 2 to 5 d postnatal to accrue ChAc activity in culture (Fig. 2) suggests that at this stage in their *in vivo* development sympathetic neurones of the SCG receive important information related to the expression of transmitter mechanisms. A critical event during the differentiation of these neurones apparently begins about 2 d postnatally; the result of this event is a commitment to being adrenergic.

Although these experiments do not rule out the possibility that preganglionic connections influence the expression of cholinergic or adrenergic mechanisms in the sympathetic neurone, available information is more supportive of the view that the target organ, containing adrenergic receptors and perhaps trophic signals (other than nerve growth factor) finally determines the appropriate neurotransmitter these neurones synthesise (ref. 11 for review). The innervation of target organs by the rat SCG is incomplete at birth. Studies involving electron microscopy¹², histochemical fluorescence¹³, norepinephrine uptake¹⁴, and changes in tyrosine hydroxylase activity¹⁵ indicate that sympathetic innervation in iris begins 2–3 d postnatally and that mature innervation in iris is not achieved until 3–4 weeks after birth; in the pineal, mature innervation is not observed until 2–3 weeks after birth^{16,17}. Our tissue culture observations suggest that a signal, most likely from the adrenergic target organ, begins its influence about the second postnatal day in the rat and that this signal ensures the commitment to adrenergic function which characterises most sympathetic neurones *in vivo*.

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Phosphorylation of acetylcholine receptor by endogenous membrane protein kinase in receptor-enriched membranes of *Torpedo californica*

THE molecular basis of synaptic transmission is a fundamental problem in neurobiology. Although neurotransmitters produce striking changes in post-synaptic ion permeability, little is known about the molecular events following binding of neurotransmitter to its receptor. It has been postulated that the effects of neurotransmitters on post-synaptic membranes are mediated by changes in protein phosphorylation^{1,2}. There has been no direct evidence for neurotransmitter-dependent changes in protein phosphorylation in membranes prepared from innervated tissues, however. We investigated this problem using an acetylcholine receptor-enriched membrane fraction prepared from the electric organ of *Torpedo californica* and found that several membrane polypeptides are phosphorylated when this fraction is incubated with γ -³²P-ATP and 100 mM K⁺ (ref. 3). In particular, we demonstrated that a polypeptide of 65,000 molecular weight (MW) is phosphorylated and that phosphorylation of this and other polypeptides is inhibited by cholinergic ligands. A 65,000 MW polypeptide in the same membrane preparation also reacted with antibody prepared against the purified acetylcholine receptor. This suggested that a component of the acetylcholine receptor itself could be phosphorylated by an endogenous membrane protein kinase. Here we present proof that the 65,000 MW component of the acetylcholine receptor is phosphorylated by the endogenous membrane protein kinase.

Membranes enriched 15-fold in acetylcholine receptors were prepared from the electric organ of *T. californica* as described previously³. Figure 1a shows the polypeptide composition of the acetylcholine receptor-enriched membranes. This fraction contains endogenous protein kinase activity which maximally phosphorylates four polypeptides in the presence of 5 μ M ATP, 100 mM K⁺ and 0.005% Triton X-100 (ref. 3). To prove that a component of the acetylcholine receptor is phosphorylated by the endogenous protein kinase, phosphorylation of the acetylcholine

receptor was studied using two-dimensional immunoelectrophoresis as described by Converse and Papermaster⁴. Acetylcholine receptor-enriched membranes were incubated with γ -³²P-ATP and electrophoresed in sodium dodecylsulphate (SDS) polyacrylamide gels as described in Fig. 1. The gels were cut into longitudinal strips along the sample wells; one gel was stained for protein, another was subjected to immunoelectrophoresis at right angles into Agarose containing anti-acetylcholine receptor serum. Polypeptides derived from the acetylcholine receptor which are present in optimal concentrations form immunoprecipitates with the anti-acetylcholine receptor antibody and are recognised as 'rockets.' Figure 1b shows one major Coomassie blue stained 'rocket' which corresponds to the immunoprecipitate formed by the reaction of the antiserum with the 65,000 MW polypeptide of the acetylcholine receptor. Control gels run against pre-immune serum show no 'rockets.' Autoradiography of the dried gel could be used to determine whether the immunoelectrophoretic 'rocket' is radioactive. Figure 1c demonstrates that the same polypeptide precipitated by anti-acetylcholine receptor serum contains ³²P. Two other components of the acetylcholine receptor also react with antiserum and contain minor amounts of ³²P. Their polypeptide-antiserum concentration ratios are, however, not optimal for adequate resolution of the corresponding 'rockets.' This experiment provides firm evidence that the 65,000 MW component of acetylcholine receptor is phosphorylated by an endogenous membrane protein kinase present in receptor-enriched membranes. The major immunoprecipitate consists only of antibody against the acetylcholine receptor and the 65,000 MW component of the receptor⁴. Therefore, ³²P

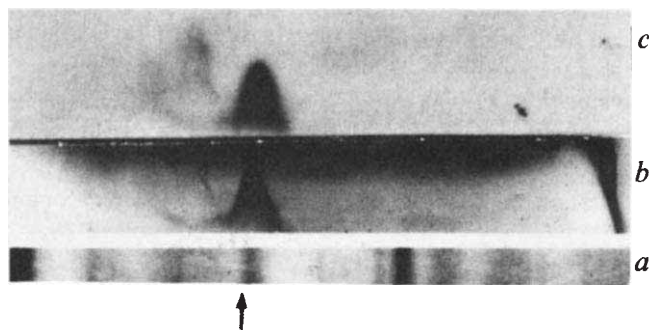


Fig. 1 Two-dimensional immunoelectrophoresis of acetylcholine receptor-enriched membranes. Acetylcholine receptor-enriched membranes were prepared from the electric organ of *T. californica* as previously described³. Membranes were incubated for 0.5 min at 0 °C in γ -³²P-ATP (1-2 μ Ci per tube), 0.25 mM EGTA, 10 mM MgAc₂, 0.0625 M Tris-HCl pH 6.8 and 100 mM KCl. The reaction was stopped by the addition of SDS to a final concentration of 4% and mercaptoethanol was added to a final concentration of 5%. Electrophoresis of 50 μ g and 75 μ g of membrane protein in SDS polyacrylamide slabs was carried out according to the method of Laemmli⁹ with 8% acrylamide in the running gel and 3% in the stacking gel. The slabs were run overnight at 8 mA per gel. The gels were then cut into longitudinal strips. *a*, The strip containing 50 μ g of membrane protein was simultaneously fixed and stained for protein for 2 h in 25% isopropanol, 10% acetic acid and 0.2% Coomassie blue and destained overnight in 25% isopropanol, 10% acetic acid. *b*, *c*, The strip containing 75 μ g of membrane protein was electrophoresed at right angles to the original direction into two layers of Agarose⁴, the first containing 1.5% Lubrol PX (Sigma) and the second containing 16% goat anti-acetylcholine receptor antiserum. Antiserum was prepared against purified *T. californica* receptor as previously described³. Immunoelectrophoresis was run at a constant voltage of 53 V (measured across the gel) for 2 h at 4 °C. The acrylamide strip and the Agarose layer were then removed to minimise electroosmotic effects. Agarose containing buffer⁴ was used to replace the removed layers. Immunoelectrophoresis was resumed for an additional 4 h at 53 V. The Agarose gels were then incubated overnight to remove unreacted serum, in 10 mM NaPO₄ pH 7.4, containing 150 mM NaCl, dried and stained as described above (*b*). The gels were subjected to autoradiography on Kodak NS2T X-ray film for 5 weeks (*c*). Arrow represents MW of 65,000.

present in this 'rocket' must be derived from a phosphorylated component of the acetylcholine receptor.

In summary, we have found that the acetylcholine receptor is a substrate for an endogenous membrane protein kinase. We have recently demonstrated that phosphorylation of receptor-enriched membranes is regulated by cholinergic ligands³. Moreover, phosphorylation of the receptor may account for the observation that the acetylcholine receptor exists in two forms with different isoelectric points^{3,6}. Taken together these findings suggest that phosphorylation of the receptor may play an important role in post-synaptic events. Rhodopsin also is an extensively purified membrane protein which undergoes phosphorylation regulated by its physiological stimulus; this molecule is phosphorylated in the presence of light and ATP^{7,8}. It has been suggested^{7,8} that phosphorylation of rhodopsin might be related to a decrease in Ca²⁺ ion permeability. Furthermore, it has been shown that light-stimulated phosphorylation of rhodopsin is due to a direct conformational change of the visual pigment and not due to an effect of light on protein kinase activity. By analogy, changes in the phosphorylation of the acetylcholine receptor might be the result of conformational changes in the receptor protein induced by cholinergic ligands³. Phosphorylation could have a role in regulation of ion permeability or in other postsynaptic phenomena such as desensitisation, maintenance of the receptor at the synapse, trophic effects of innervation, and appearance of extra-junctional receptors after denervation. The finding that the acetylcholine receptor itself is phosphorylated provides a new avenue for investigation of these possibilities.

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In vitro phosphorylation of the acetylcholine receptor

In a study of the properties of the acetylcholine receptor protein (AChR) solubilised from extrasynaptic and subsynaptic areas of rat diaphragm, Brookes and Hall¹ reported differences in the behaviour of these receptors on isoelectric focusing and concluded that extrasynaptic and subsynaptic receptors "have similar properties but are still distinct molecules". Applying the same technique to crude detergent extracts of membrane fragments from *Electrophorus electricus* electric organ², we also found two forms of AChR with isoelectric points similar to those reported by Brookes and Hall. Furthermore a catalytic inter-conversion between these two forms was achieved by incubating *in vitro* the membrane extract in the presence of 100 mM NaCl or 100 mM NaF. This finding was interpreted as resulting from a phosphorylation-

dephosphorylation of the AChR. In agreement with this interpretation, it has been shown³ that endogenous protein kinase and phosphoprotein phosphatase activities are indeed present in membrane fragments isolated from the electric organ of *E. electricus*. We report here that in detergent extracts of these membrane fragments, a phosphorylation of AChR takes place when incubated in the presence of γ -³²P-ATP, Mn²⁺ and the endogenous protein kinases.

Membrane fragments were prepared by homogenising fresh or frozen electric tissue (40 g) into 80 ml of PNM (10⁻⁴M phenylmethylsulphonylfluoride, 0.02% NaN₃, 0.014 M 2-mercaptoethanol in H₂O, pH 6.2) for 5 min at 0 °C using a Virtis blender at medium speed. This homogenate was centrifuged for 30 min, 20,000g at 4 °C and the pellet resuspended in 20 ml PNM. The extraction of AChR was carried out by gently stirring the resuspended membrane fragments in the presence of 5% Triton X-100 for 60 min at room temperature. The suspension was then centrifuged at 100,000g for 60 min, at 4 °C and the supernatant ('detergent extract') collected and kept at 4 °C. The AChR was assayed with ³H- α -toxin (12.4 Ci mmol⁻¹) from *N. nigricollis*⁴; 80-100 pmol of AChR were present per ml of detergent extract. Phosphorylation of AChR was carried out by incubating the detergent extract for 30 min at room temperature in the presence of 100 mM Tris, 10⁻³M ouabain, 10 mM MnCl₂, 100 mM NaF and 4 μ M γ -³²P-ATP (16 Ci mmol⁻¹ purchased from Amersham) at pH 7.4 in a total volume of 1 ml. This mixture was then applied on top of a Sephadex G-50 column (46 cm $\times$ 2.2 cm) equilibrated and eluted in 20 mM Tris, 0.1% Triton X-100, 50 mM NaF, 10⁻⁴M phenylmethylsulphonylfluoride, 0.02% NaN₃ at pH 7.4. The protein fraction eluted in the void volume was pooled into a dialysis bag and concentrated at room temperature over polyethyleneglycol powder. The content of the dialysis bag was then removed, centrifuged for 3 min at 2,000g 4 °C and the supernatant ('phosphory-

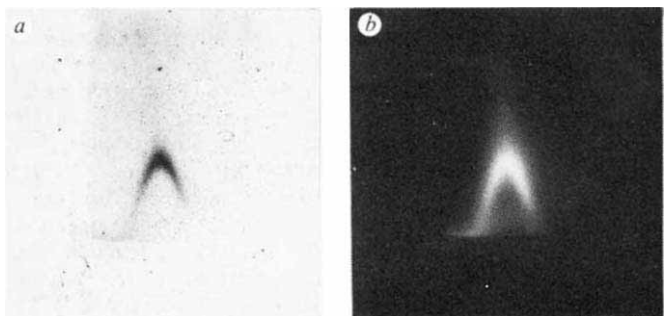


Fig. 1 Crossed-immunoelectrophoresis was carried out on Agarose gel. 15 ml of a 1% solution of Indubiose A45 in 24.5 mM veronal, 0.48 mM calcium lactate, 2 mM NaN₃, 1% Triton X-100, 73 mM Tris-HCl, pH 8.6, were poured on a 10 cm $\times$ 8.5 cm glass plate. After congelation, a 3 cm $\times$ 3 cm gel slab was then cut out in the middle of the plate and replaced with 1.6 ml of the above gel solution containing 25-50 μ l of anti-AChR serum. A 3 mm $\times$ 6 mm well is then punched in the lower left of the antibodies containing gel for the application of 10-30 μ l of antigen solution. The electrophoresis in the first dimension is carried out at 15 °C for 45 min at 10 V cm⁻¹ in the above Tris-veronal buffer pH 8.6. Anode to the right. The second dimension is carried out at 15 °C for 16-20 h at 2 volts cm⁻¹ with the anode at the top. After electrophoresis, the gel is pressed under several layers of blotting paper and a weight of about 10 g cm⁻², washed several times in 20 mM Tris-HCl, 0.1 M NaCl, 0.1% Triton X-100, pH 7.4, pressed again and finally washed in H₂O and dried. The dry gel is stained with 0.5% Coomassie blue in ethanol-acetic acid-H₂O (4.5/1/4.5) and destained in ethanol-acetic acid-H₂O (4.5/1/4.5). The destained gel is washed in H₂O and dried. It is then placed in contact with a Kodak Kodirex film. The autoradiogram is developed 1-2 d later. *a*, Coomassie blue staining of the precipitation line(s) between anti-AChR antibodies and ¹²⁵I- α -bungarotoxin-AChR complex. 20 μ l of detergent extract containing 1.8 pmol AChR and 1.5 pmol of ¹²⁵I- α -bungarotoxin (238 Ci mmol⁻¹) were applied. Anti-AChR antiserum was at a density of 5 μ l of serum per cm². *b*, autoradiograph of (*a*) after 24-h exposure.

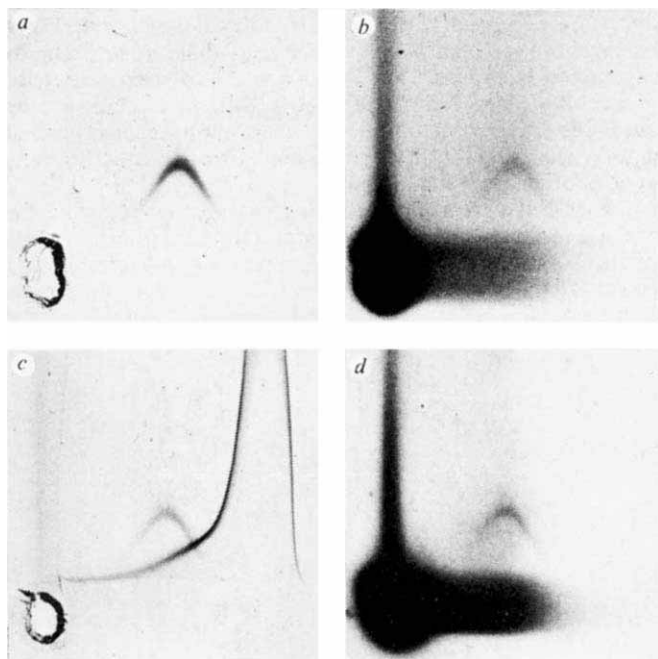


Fig. 2 Crossed-immunoelectrophoresis. Conditions as described in Fig. 1. *a*, Anti-AChR antibodies and phosphorylated detergent extract. 1.1 pmol of AChR in 18 μ l were applied. Anti-AChR antiserum was at a density of 4.5 μ l of serum per cm^2 . *b*, Autoradiograph of (*a*) after 24-h exposure. *c*, Anti-bovine serum albumin antibodies and bovine serum albumin (large peak) and anti-AChR antibodies and phosphorylated detergent extract. 1.3 pmol of AChR and 15 pmol bovine serum albumin were applied in 20 μ l. Anti-AChR and anti-bovine serum albumin antisera were respectively at a density of 3.3 and 0.55 μ l of serum per cm^2 . *d*, Autoradiograph of (*c*) after 48-h exposure.

lated detergent extract') was removed and kept at 4 °C. Rabbit sera (anti-AChR) were raised against Triton X-100 extracted AChR purified from *E. electricus* membrane fragments by affinity chromatography⁸. The purified AChR was analysed by dodecylsulphate polyacrylamide gel electrophoresis and found to be composed of three polypeptides of molecular weights of 43,000, 49,000 and 59,000.

To demonstrate the phosphorylation of the receptor protein, crossed-immunoelectrophoresis⁹ was used to identify and separate the AChR from the bulk of the proteins and lipids present in the detergent extract. The anti-AChR antiserum was first assayed against an aliquot of detergent extract preincubated with ¹²⁵I- α -bungarotoxin (238 Ci mmol^{-1}). Only one precipitation line was observed both by Coomassie blue staining and by autoradiography (Fig. 1*a, b*). When the phosphorylated detergent extract underwent crossed-immunoelectrophoresis in a 1% Agarose gel containing anti-AChR antibodies, again only one precipitation line was seen both by Coomassie blue staining and by autoradiography (Fig. 2*a, b*). A nonspecific absorption of phosphorylated proteins on the AChR-anti AChR precipitate could, however, be the cause of the line seen by autoradiography (Fig. 2*b*). To test this possibility, a mixture of 'phosphorylated detergent extract' and bovine serum albumin was submitted to crossed-immunoelectrophoresis in a gel containing both anti-AChR and anti-bovine serum albumin antibodies. This time, two precipitation lines were stained by Coomassie blue: one corresponded to the AChR, the other to bovine serum albumin. Only the line characteristic of the AChR was detected by autoradiography, however, (Fig. 2*c, d*), indicating without ambiguity that the AChR had been phosphorylated.

To determine which polypeptide chains of the purified AChR had been phosphorylated, AChR-anti-AChR precipitates obtained from crossed immunoelectrophoresis were cut out of agar-coated plates, solubilised in 2% sodium dodecylsulphate, 3% mercaptoethanol, 0.062 M Tris at

pH 6.8, and analysed by dodecylsulphate polyacrylamide gel electrophoresis and autoradiography. Only one polypeptide of apparent molecular weight 48,000 could be clearly detected on the densitogram obtained after scanning of the autoradiograph (Fig. 3).

The AChR protein purified from *E. electricus* gives two (ref. 5) or three (ref. 7 and A.S., unpublished) bands by polyacrylamide gel electrophoresis in sodium dodecylsulphate but only one of them corresponding to a polypeptide chain of apparent molecular weight $43,000 \pm 3,000$ is labelled by ³H-MBTA⁷ or ³H-MPTA (A.S., unpublished), two covalent affinity labels of the acetylcholine receptor site. Interestingly, it is not this chain which is phosphorylated *in vitro* but that of apparent molecular weight $48,000 \pm 3,000$.

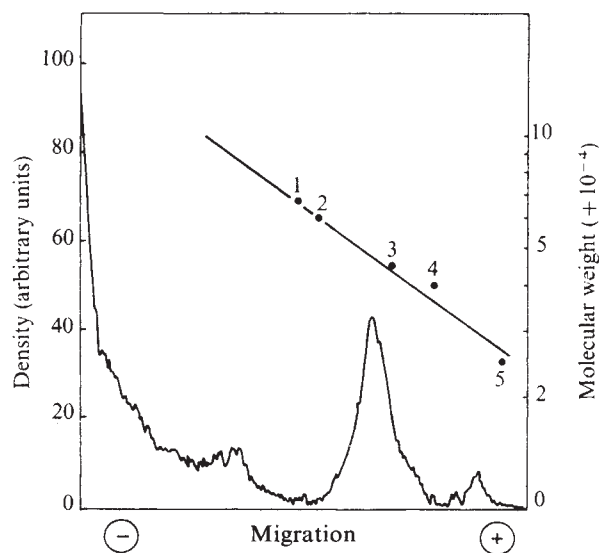


Fig. 3 Densitogram obtained from the autoradiograph of precipitates between anti-AChR antibodies and phosphorylated detergent extract after sodium dodecylsulphate polyacrylamide gel electrophoresis. Precipitates from several immunoelectrophoresis were cut out from dried Agarose plates after Coomassie blue staining and solubilised in a buffer containing 0.062 M Tris-HCl, 2% sodium dodecylsulphate, 3% 2-mercaptoethanol at pH 6.8. Electrophoresis was carried out on a 10% acrylamide slab gel of 1.5-mm width following the method of Laemmli¹¹ modified by Anderson and Gesteland¹². The gel was stained for protein with Coomassie blue, destained, washed in H₂O, dried and placed in contact with Kodak Kodirex film. The film was developed after 120 h. The densitometric scanning of the autoradiograph was carried out using a Vernon scanner and the optical density was expressed in arbitrary units. The following proteins were used as markers for molecular weight determination; 1, bovine serum albumin; 2, catalase; 3, albumin; 4, aldolase; 5, chymotrypsinogen. The band of 48,000 molecular weight could not be detected by Coomassie blue staining.

As the *in vitro* phosphorylation-dephosphorylation of the AChR takes place under conditions close to those needed for the interconversion between the two 'isoelectric' forms of the AChR, one may relate these reactions to at least two different regulatory mechanisms. (1) For instance, the 48,000 molecular weight polypeptide is the ionophore and its transport properties are regulated by phosphorylation-dephosphorylation reactions as proposed by Greengard⁹. The differences in isoelectric points between extra- and subsynaptic receptors would simply result from the fact that only the subsynaptic AChR is activated by the neurotransmitter and therefore phosphorylated. (2) In relation with the theory on selective stabilisation of developing synapses¹⁰, the phosphorylation of the AChR could be an element of the complex 'stabilisation reaction' postulated to cause the interconversion of the labile and mobile form of the AChR present in extrasynaptic areas into its stable and immobile form in the mature subsynaptic membrane. If this reaction was indeed taking place in *E. electricus* electric organ despite the fact that it is not an embryonic

system, then the phosphorylated 48,000 molecular weight chain would not necessarily represent the ionophore but could for instance be involved in the aggregation of the receptor oligomer and/or its protection against proteolysis.

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Association of DNA synthesis with the nuclear membrane in sea urchin embryos

THERE is considerable evidence implicating the cell membrane as the site for DNA replication in prokaryotic systems^{1–3}. Several laboratories have considered the possibility that the nuclear membrane may serve a similar role in eukaryotes. This model has been supported by biochemical studies in various eukaryotic cell systems^{4–8}. But, autoradiographic analyses in mammalian cells, that previously indicated that DNA synthesis was initiated at the nuclear membrane^{9,10}, now provide the strongest evidence against an involvement of the nuclear membrane in DNA synthesis^{11–13}. The sea urchin system presents several advantages for studies of DNA synthesis. On fertilisation a population of dormant unfertilised eggs begins a series of very rapid cellular divisions, the first three of which are naturally synchronous. Biochemical studies with this system have suggested that rapid replication of DNA occurs at a nuclear membrane site and that DNA may be associated with the site only during the period of DNA synthesis¹⁴. We therefore decided to undertake an autoradiographic study in the sea urchin embryo. The results presented here strongly support the role of the nuclear membrane in the synthesis of DNA.

We examined DNA synthesis during the S-phase immediately following first cleavage. In agreement with Hinegardner *et al.*¹⁵, we found that DNA synthesis begins in mid-telophase, a stage in which each chromosome is encased in its own double membrane vesicle, termed a karyomere (Fig. 1a). The grain distribution in embryos pulsed at this time for either 30 or 60 s showed a peripheral labelling pattern within each karyomere (Fig. 1a, b). A quite different distribution of grains was obtained if, instead of a short exposure to thymidine, the embryos were incubated for a longer period. After 5 min of labelling (which in this system is more than $\frac{1}{3}$ of the S-period), there was an increased number of grains in the central area of the karyomere (Fig. 1c). Although there was still dense incorporation at the periphery, the result demonstrates that chromatin is not confined to the periphery.

It is important to make certain that the grains represent incorporation of labelled nucleotide into DNA. Thymidine is

incorporated into acid precipitable material which is resistant to digestion in alkali, and is DNase labile. Also embryos exposed to label before the S-phase, and even into early telophase, showed no radioactive incorporation when assayed by autoradiography. Finally, there is virtually no background of grains appearing in the cytoplasmic areas even after long periods of incorporation.

Figure 1 shows that increased exposure to label during the S-phase results in an increase in grains appearing in the centre of the karyomere and subsequent nuclei. This indicates that either (1) the location of DNA relative to the membrane is stable and the replication machinery begins synthesis at the

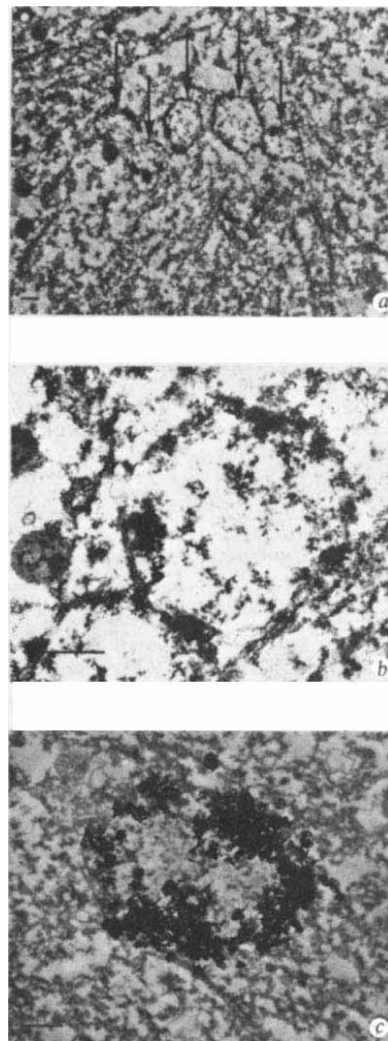


Fig. 1 Labelling of karyomeres for a, 30 s; b, 90 s, and c, 5 min following the start of S phase. Gametes from the Pacific sea urchin, *Strongylocentrotus purpuratus* were obtained by injecting 0.55 M KCl into the coelomic cavity to induce shedding. The eggs were washed, fertilised, and incubated at 16 °C as previously described¹⁴. At 100 min after fertilisation (the start of the second DNA synthetic period¹⁵) radioactive thymidine (methyl-³H; New England Nuclear; 50 Ci mmol⁻¹) was added to a final concentration of 5 $\mu\text{Ci ml}^{-1}$. Samples were removed at the times indicated and the pulse stopped by the addition of an equal volume of Karnofsky's fixative. After settling, the labelled embryos were resuspended in fresh fixative, allowed to sit for 2 h, and postfixed for an additional hour in 1% osmium tetroxide. Following ethanol dehydration, the fixed embryos were embedded in Epon resin and sectioned on an LKB ultramicrotome. Purple/gold sections were removed from the air/water interface using Formvar coated copper grids and stained with uranyl acetate and lead citrate, then carbon coated under vacuum. The grids were coated with Ilford L-4 emulsion (Poli-sciences) using the technique of Stevens²⁴ and stored at -60 °C for 14 d–6 months. After development, the grids were routinely restained with lead citrate. Micrographs were made using a Philips 300 electron microscope. The bar in each figure represents 1 μm .

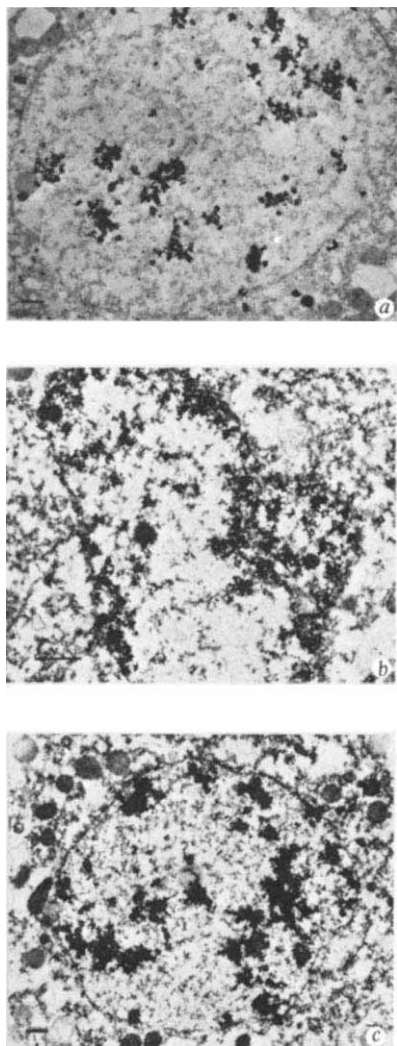


Fig. 2 Distribution of grains in nuclei labelled for various periods of time. *a*, Nucleus of embryo labelled continuously for 20 min beginning at 100 min after fertilisation. *b*, Embryos pulsed for 30 s, washed twice in normal sea water, and then resuspended in normal sea water containing 200 μ M unlabelled thymidine. The chase period was for 4.5 min. *c*, Embryos pulsed for 30 s and chased for 20 min in cold thymidine as in *b*. Embryos were labelled with 3 H-thymidine, fixed, and prepared for autoradiography as described in Fig. 1.

periphery and moves into the central area, or (2) nascent DNA is displaced from a stable site of replication at the periphery and moves into the centre. To distinguish between these two possibilities we looked at the distribution of continuously labelled DNA after the completion of the S-phase and the fate of the DNA synthesised during early S using a pulse-chase analysis. A typical result of the first study is shown in Fig. 2*a*. The pattern of grains seen in nuclei after 20 min of labelling indicates a concentration of radioactive DNA within the central areas and little or none present at the periphery (Fig. 2*a*). Under such conditions the DNA is uniformly labelled and the embryos are no longer in the S-phase. This shows that once replication is completed, virtually all of the DNA is displaced away from the membrane region; and that most of the DNA is not permanently associated with the membrane. A pulse-chase analysis supports these conclusions. Nuclei of embryos pulsed during the first 30 s of the S-phase and chased with cold thymidine for either 4.5 min or 19.5 min are compared in Fig. 2*b* and *c*, respectively. After a short chase, karyomeres typically showed a trend towards more homogeneous labelling. While there is still a large percentage of grains present at the periphery, there appears to be a significant increase in central label. This trend in apparent relocalisation of grains is much clearer in Fig. 2*c* which shows that after a long chase period the DNA

labelled during a short pulse in early S is located predominantly in the central region of the nucleus and does not maintain a stable location at the periphery. Taken together, these results support the second possibility presented above: once replication is completed, the nascent DNA is displaced away from a stable site of replication located at the nuclear membrane.

We attempted to quantify our results using a histogram analysis (defined in legend of Fig. 3) as used by Huberman *et al.*¹². Figure 3 compares histograms derived from embryos labelled for pulse, long term, and pulse-chase periods. All the embryos labelled for short periods have central activities of less than 1.0 (Fig. 3*a*). The mean central activity in these embryos is 0.4 indicating that the peripheral areas are enriched with many more grains than would be expected if the nascent DNA were labelled homogeneously in all areas of the karyomere. In contrast, Fig. 3*b* shows that the majority of nuclei labelled continuously through the complete S-phase have central activities greater than 1.0 indicating a general lack of peripheral label. This result also indicates that most of the DNA is located in the central region of the nucleus. When embryos are pulsed

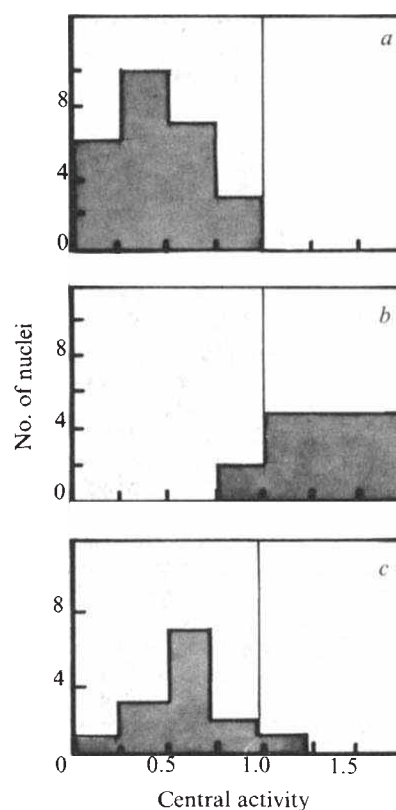


Fig. 3 Distribution of grains in nuclei of sea urchin embryos after various labelling conditions. Histogram analysis of central activities in cells labelled as in Fig. 2 for: *a*, pulse (30–100 s); *b*, continuously labelled (20 min); *c*, pulse/chase (labelled 30 s then chased for 4.5 min). Central activity is defined as the ratio of the fraction of grains which are central to the fraction of area which is central. A central activity of 1.0 indicates a homogeneous distribution of grains throughout the nucleus; ratios of < 1.0 signify a preferential labelling of the peripheral area. A central activity of > 1.0 indicates a preferential localisation of grains away from the periphery. Only karyomeres/nuclei with four or more grains were scored. Grains outside the nuclear membrane, regardless of their proximity, were not counted. For karyomeres, a rim 0.5 μ m in width was considered as the peripheral area. For nuclei, the peripheral area was a rim 1.5 μ m in width. This differentiation in width was made so that the percentage of area considered central in all cases remained approximately the same. To determine the ratio of area, paper tracings of the micrographs were made, the peripheral area measured and cut, and the fraction determined by the weight of the paper. The paper rim was then placed on the micrograph to determine the fraction of grains that were central. After the 4.5 min chase in (*c*) many grains are associated with the periphery probably because the large internal nucleotide pools¹⁶ are not completely diluted during such a short chase.

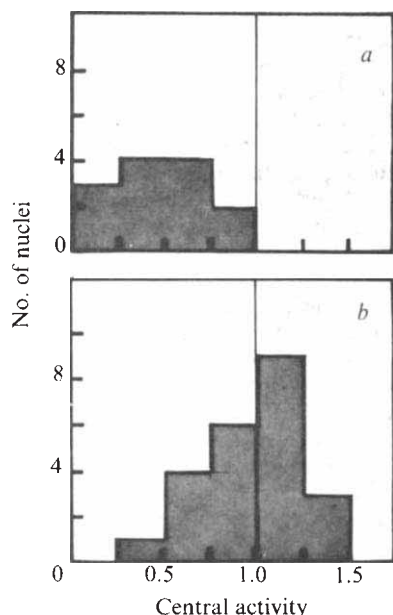


Fig. 4 Histogram analysis of grain distribution in late S phase labelled nuclei. *a*, Nuclei labelled for 30 s. *b*, Nuclei labelled for 90 s. Embryos developing at 16 °C were labelled beginning at both 108 and 112 min after fertilisation. After radioactive precursor incubation, samples were removed, fixed, and prepared for autoradiography as described in Fig. 1. Results from the short and long term labelling at both the 108 and 112 min time points were virtually the same and were combined.

and then chased for a short period the values for the central activities are shifted toward 1.0 (Fig. 3c), a trend away from the strong peripheral labelling and towards a more homogeneous labelling pattern.

Finally, we tested whether nascent DNA synthesised in the late S-phase is also localised in the peripheral region (Fig. 4). Embryos at 110 min after fertilisation were pulsed for 30 or 60 s. The S-phase in these embryos is at least two-thirds completed and the nuclei are entirely reformed. At this time the incorporation of exogenous precursor seems to occur much more slowly, based on the number of grains detected per nucleus in comparison to embryos in the early S-phase. But, like the patterns in early S-phase, the distribution of grains in nuclei pulse-labelled during late S indicates that incorporation is still taking place largely at the periphery (Fig. 4a). When these embryos are allowed to incorporate label for longer periods the central activity values are distributed around 1.0 (Fig. 4b) indicating a shift of grains away from the membrane.

The autoradiographic evidence presented here supports our previous biochemical studies indicating that the nuclear membrane may be involved with the temporal regulation of DNA replication in the sea urchin¹⁴. An enrichment of radioactive DNA in a presumptive DNA-membrane fraction was also found when we examined pulse-labelled nuclei of cultured mouse fibroblasts (3T3 cells)¹⁷; however an autoradiographic study in this system has not been reported. It is difficult to resolve our results with the autoradiographic results of others using mammalian culture cells. A possible explanation may be that autoradiographic analysis cannot distinguish between replication and repair synthesis of DNA, two activities known to occur in the S-phase nucleus. In the naturally synchronous sea urchin embryo where, during the S phase 1.8 pg of DNA is replicated in 12–15 min (ref. 15) semi-conservative replication may be assumed to be the principal synthetic activity. In mammalian culture cells, the period of time during which DNA is replicated is relatively long and it is now thought that replication events are initiated sporadically during this time¹⁸. This suggests that replication is not a continuous event during the DNA synthetic period and may be characterised by many starts and stops^{19,20}. Thus, a pulse label of cells from either

synchronised or unsynchronised cultures may result in the incorporation of exogenous precursor due to both repair and replication. Such a situation would confuse the interpretation of autoradiographs in determining subnuclear sites of replication.

In mammalian systems there is general agreement that during the late stage of the S-phase DNA synthesis occurs preferentially near the membrane^{9,11,12}. This result is commonly considered to be due to the replication of heterochromatin which is known to be replicated late in the S-phase^{21,22} and to be located near the membrane^{21,12,23}. In our studies the pulse-labelled DNA is preferentially located at the membrane both in the early and late S-phase. Furthermore it is clear that in this system virtually all of the DNA is located in the central region of the nucleus (Figs 2a, 3b). The simplest conclusion is that DNA synthesis occurs at the nuclear membrane and is relocated away from the membrane.

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Primary structure of porcine relaxin: homology with insulin and related growth factors

HISAW and co-workers^{1,2} first described a factor from sow corpora lutea that caused relaxation of the pubic symphysis in oestrogen-primed guinea pigs. Subsequent studies have shown that the active substance, termed relaxin, is a low molecular weight peptide hormone. Its presence has been demonstrated during pregnancy in female pigs, guinea pigs, rabbits, mice and rats^{3,4}, and, more recently, in humans⁵. Its actions include dilatation and softening of the cervix, inhibition of uterine contractions and relaxation of the pubic symphysis and other pelvic joints. Relaxin, which has a molecular weight of approximately 6,000, consists of two non-identical chains joined by disulphide bridges⁶. We have now determined, and report here, the complete amino acid sequence of porcine relaxin.

Relaxin was purified from pregnant sow ovaries⁶; biological activity was localised by the mouse pubic symphysis bioassay⁷. Relaxin fractions used for structural studies had biopotencies in the range 1,100–1,500 guinea pig units (GPU) per mg relative to reference preparation NIH-R-P1 (potency 442 GPU mg⁻¹).

As a preliminary to sequence analysis, relaxin was reduced with dithioerythritol in urea or guanidine solutions

A CHAIN

H - Arg - Met - Thr - Leu - Ser - Glu - Lys - Cys - Cys - Gln - Val - Gly -
Cys - Ile - Arg - Lys - Asp - Ile - Ala - Arg - Leu - Cys - OH

Fig. 1 Amino acid sequences of A and B chains of relaxin. PCA, pyrrolidone carboxylic acid.

B CHAIN

PCA - Ser - Thr - Asn - Asp - Phe - Ile - Lys - Ala - Cys - Gly - Arg - Glu -
Leu - Val - Arg - Leu - Trp - Val - Glu - Ile - Cys - Gly - Ser - Val - Ser -
Thr - Trp - Gly - Arg - OH

and alkylated with ^{14}C iodoacetic acid⁸. A number of methods were investigated for chain separation. During attempts to use ion exchange chromatography for this purpose it was observed that the longer chain (30 amino acids, designated 'B') was insoluble in the pH range 4-7, whereas the shorter chain (22 amino acids, designated 'A') remained soluble. Thus chain separation could be achieved simply by low speed centrifugation of the reduced carboxymethylated preparation. Schwabe *et al.* have described a similar procedure⁹.

The amino acid sequence of the A chain (Fig. 1) was established in three separate experiments by manual Edman degradation¹⁰. After 21 cycles the residual sample was subjected to amino acid analysis. Free carboxymethylcysteine was identified; thus this residue occupies the C terminal position in the A chain.

Sequence analysis of the B chain proved more difficult since the N terminus was found to be resistant to Edman degradation. Removal of the N-terminal blocking group could be achieved, however, by a pyrrolidone carboxyl peptidease¹¹. Thus the terminal residue was identified as pyroglutamic acid. Edman degradation was then carried out for 16 cycles on the deblocked B chain.

The remainder of the sequence (Fig. 1) was established by manual Edman degradation of the peptide Leu¹⁷ . . . Arg³⁰ isolated from a tryptic digest of carboxymethylated B chain. Confirmatory studies were carried out through Edman degradation and carboxypeptidase digestion of tryptic peptides isolated by preparative paper electrophoresis. Schwabe *et al.*⁹ have previously proposed a sequence for the A chain of relaxin identical to ours except that they find residue 10 to be glutamic acid whereas in three different preparations we clearly identified glutamine at this position. Deamidation of their material during isolation or sequence analysis could explain this discrepancy.

There are several features of the relaxin structure that deserve comment. The marked structural similarity to insulin is evident. Thus, relaxin consists of two dissimilar peptide chains of 22 and 30 residues respectively. The number and distribution of half-cystine residues are identical in the two hormones. Neither has free sulphhydryl groups. We have not yet rigorously established the pairing of the three disulphide bonds in relaxin; however, preliminary evidence suggests that the arrangement is identical to that of insulin, with two interchain bridges and an intra-

chain bridge in the relaxin. A chain linking the half-cystines at positions 8 and 13.

Amino acid sequence homology between relaxin and insulin is more obvious in the B than in the A chains (Figs 2 and 3). Superficially the A chain sequence homology does not seem impressive apart from the exact alignment of the four half-cystine residues. But, reference to the three-dimensional structure of insulin known from X-ray crystallographic studies^{12,13} shows that there is virtually complete conservation of the residues in the insulin A chain that contribute to the hydrophobic core of the molecule. These are the half-cystine residues found in insulin at A6 and A11, together with the A2 isoleucine (leucine in relaxin) and A16 leucine (isoleucine in relaxin). Two further residues from the insulin B chain (B11 and B15 leucines) complete the hydrophobic core; both are conserved in relaxin. A further perspective appears when residues invariant in all known insulin structures¹³ are examined. These (designated with asterisks in Figs 2 and 3) comprise 10 residues in each chain. At these 20 positions, a total of 15 residues are either identical or conservatively substituted in relaxin. Within the central helical region of the insulin B chain (B9 to 19) four of the five invariant residues are identical in relaxin; the fifth (tyrosine B16) is replaced by tryptophan in relaxin. At several other positions within this sequence substitutions are seen which conserve the hydrophilic or hydrophobic nature of the residue (for example, serine B9 is replaced by arginine in relaxin; glutamic acid B13 by arginine; leucine B17 by valine; valine B18 by isoleucine). These findings suggest that important elements of the tertiary structure of insulin are also present in relaxin, and that their overall three-dimensional structures may be quite similar.

An important difference is seen in the B chain where the zinc-binding histidine residue found at position B10 in almost all insulins is replaced by glutamic acid in relaxin. This makes it most probable that relaxin will not bind zinc and thus will not be able to form zinc-coordinated dimers or hexamers. In this relaxin resembles guinea pig, coypu and hagfish insulins, which are stable as monomers in solution even at high concentrations.

Relaxin has arginyl residues at the N terminus of the A chain (A1) and the C terminus of the B chain (B30). This arrangement, taken with the other evidence for homology, strongly suggests that relaxin is derived from a proinsulin-like biosynthetic precursor by proteolytic cleav-

Fig. 2 Comparison of sequences of insulin and relaxin A chains. Identical residues are enclosed in rectangles. Residues related through favoured substitutions²² are enclosed in circles. Residues invariant in all known insulin structures¹³ are indicated by asterisks.

Relaxin	Arg	Met	Thr	Leu	Ser	Glu	Lys	Cys	Cys	Gln	Val	Gly
Insulin			* Gly	* Ile	Val	Glu	* 5 Gln	* Cys	* Cys	Thr	Ser	10 Ile
Relaxin	Cys	Ile	Arg	Lys	Asp	Ile	Ala	Arg	Leu	Cys		
Insulin	* Cys	Ser	Leu	Tyr	15 Gln	* Leu	Glu	Asn	* Tyr	* 20 Cys	* Asn	

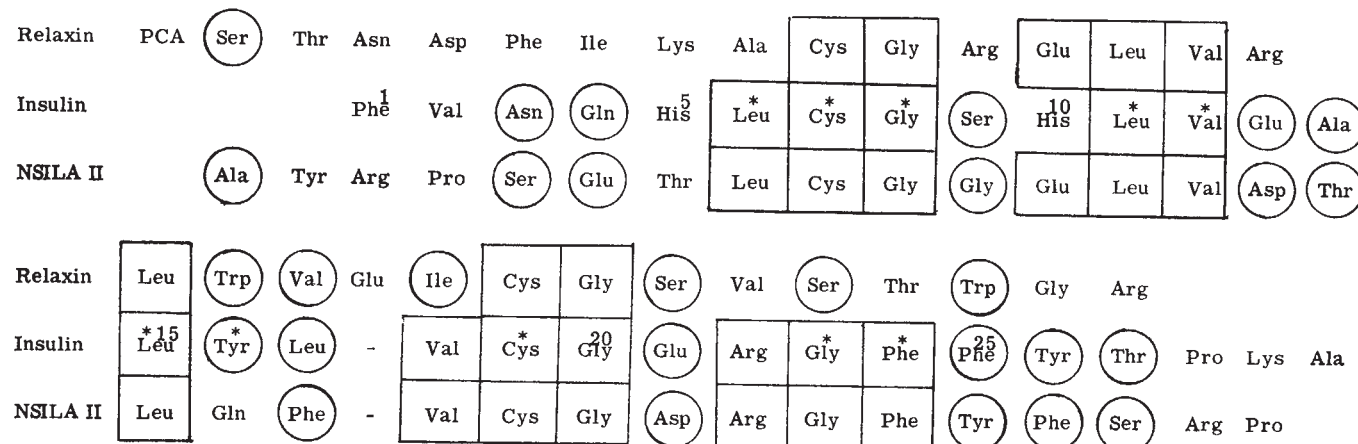


Fig. 3 Comparison of sequences of insulin and relaxin B chains and NSILA II¹⁸. Homologies and invariant residues are indicated as in Fig. 2. Gaps have been introduced in three places to improve the alignments.

age of a peptide connecting B30 to A1. Some earlier studies on relaxin also support the existence of a prohormone^{14,15}. In confirmation of earlier findings⁹ we have identified in ovarian extracts several forms of biologically active relaxin that differ slightly in net charge and amino acid composition from the peptide shown in Fig. 1. Chemical characterisation of these variants is still in progress; they could represent intermediates in the conversion of prohormone to hormone analogous to those described for insulin^{16,17} or degradation products.

The evolutionary relationship between relaxin and insulin is of considerable interest. It is likely that relaxin is related to insulin through a common ancestral polypeptide resembling proinsulin. Rinderknecht and Humbel¹⁸ have recently demonstrated a sequence homology between the insulin B chain and two polypeptides with non-suppressible insulin-like activities (NSILA I and II). These peptides are also clearly structurally related to relaxin (Fig. 3).

These homologies provide a basis for categorising relaxin as a member of a family of insulin-like growth factors with both structural and functional similarities in modulating cell growth and activity. These include nerve growth factor¹⁹, somatomedins A and C²⁰, and multiplication-stimulating factor²¹, as well as insulin and non-suppressible insulin-like activity (NSILA). Further structural studies should delineate the precise evolutionary relationships between these molecules.

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Note added in proof: Our collaborators on the X-ray crystallography of relaxin (Drs Neil Isaacs and Guy Dodson of the University of York) have been able to fit the residues between the disulphide bridges of both chains of relaxin (A8-A22 and B10-B22) into the two-zinc insulin structure without producing significant distortion. Schwabe *et al.*²³ have proposed a sequence for the relaxin B chain that differs slightly from our own at the C terminus. Whether this discrepancy is due to technical problems or to the known C terminal heterogeneity of relaxin (see text) is unclear at present.

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***In vivo* photoinactivation of *Escherichia coli* ribonucleotide reductase by near-ultraviolet light**

NEAR-ULTRAVIOLET light (320-400 nm) is lethal to bacteria¹, yeast cells² and mammalian cells³. A large body of evidence correlates cell death and survival with the induction and repair of pyrimidine dimers in the DNA of cells irradiated by far-ultraviolet light (200-300 nm) (refs 4-7). The induction of pyrimidine dimers has also been demonstrated by 365-nm near-ultraviolet light but at 7×10⁵-fold lower efficiency than by 254-nm far-ultraviolet light⁸. With the exception of certain *Escherichia coli* strains with multiple deficiencies in DNA repair^{9,10} the small number of pyrimidine dimers produced by 365-nm near-ultraviolet light does not account for a significant fraction of the biological damage either in terms of lethality¹¹ or the inactivation of transforming DNA¹². The killing of cells by near-ultraviolet light is oxygen dependent whereas that by far-ultraviolet light is oxygen independent^{5,13}. The operational distinction between far- and near-ultraviolet light based on oxygen dependence of lethality coincides with the upper limit of ultraviolet light absorption by the

Table 1 Effect of near-ultraviolet irradiation on the activity of RDP-reductase, β -galactosidase and malate dehydrogenase

Dose (erg mm ⁻² × 10 ⁻⁴)	Activity remaining (%)		
	RDP- reductase	β - galactosidase	Malate dehydrogenase
0	100	100	100
3.0	35.4	—	—
7.5	24.7	—	—
15	3.5	99.5	97.6

E. coli TAU-bar was grown in glucose salts medium¹⁹. Mid exponential phase cultures were resuspended in 0.8% saline supplemented with 0.5 g l⁻¹ KCl and 0.4 g l⁻¹ CaCl₂ at 1.0 × 10¹⁰ cells per ml. Saline suspensions were irradiated at a dose rate of 3.0 × 10³ erg min⁻¹ mm⁻² for 10, 25 and 50 min with aeration at 37 °C or were aerated at 37 °C without irradiation. RDP-reductase was assayed by the method of Warner²⁰ in whole cells made permeable to nucleotides by ether treatment. β -galactosidase and NAD-dependent malate dehydrogenase were assayed by standard spectrophotometric methods. Enzyme activities are expressed as a percentage of the unirradiated controls.

DNA at about 310 nm¹⁴ and may reflect the involvement of a primary target site other than DNA in the killing of cells by near-ultraviolet light. In *E. coli* deoxyribonucleotides are formed *de novo* by the reduction of ribonucleoside diphosphates¹⁵. Two of the components of the ribonucleoside diphosphate reductase (RDP-reductase) complex, the non-haem iron protein subunit of the RDP-reductase¹⁶ and the functionally linked flavoprotein thioredoxin reductase¹⁷ have strong absorption in the near-ultraviolet region. I show here that near-ultraviolet irradiation of *E. coli* cells selectively destroys RDP-reductase activity *in vivo* and present evidence relating the loss of RDP-reductase to the loss of cellular viability and to the inability of irradiated cells to support the replication of DNA phages.

Activity of RDP-reductase was measured in *E. coli*

Fig. 1 Percentage survival of colony forming ability as a function of near-ultraviolet dose in unsupplemented medium (□), medium supplemented with deoxyadenosine (●) or adenosine (△). Exponential phase cultures of *E. coli* TAU-bar were diluted to 5 × 10⁷ cells per ml in glucose salts medium and were irradiated at 20 °C with aeration at a dose rate of 3.0 × 10³ erg min⁻¹ mm⁻². In parallel experiments the medium was supplemented with deoxyadenosine or adenosine at 250 µg ml⁻¹. All media contained thymine at 2.0 µg ml⁻¹. Colony forming ability of irradiated cells was determined on L-agar. Irradiated glucose salts medium had no phototoxic effect when incubated with unirradiated cells.

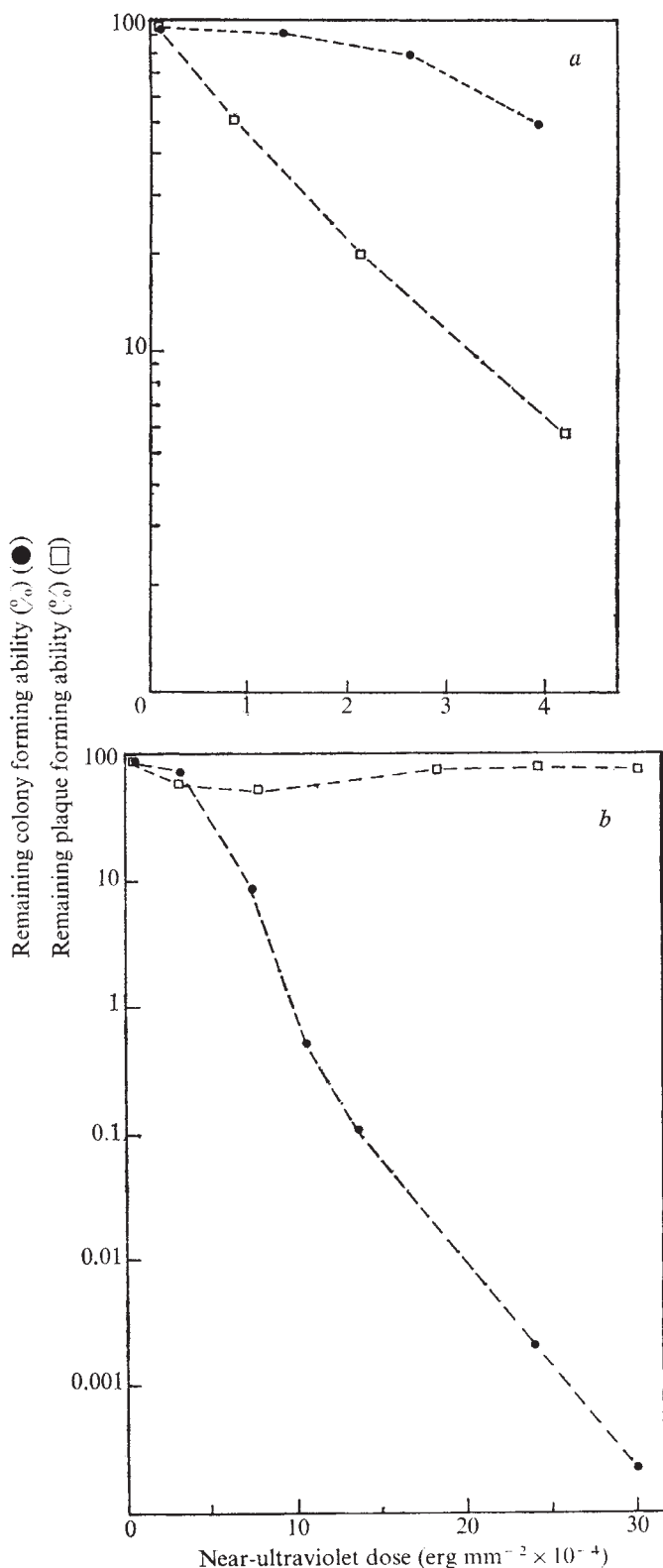
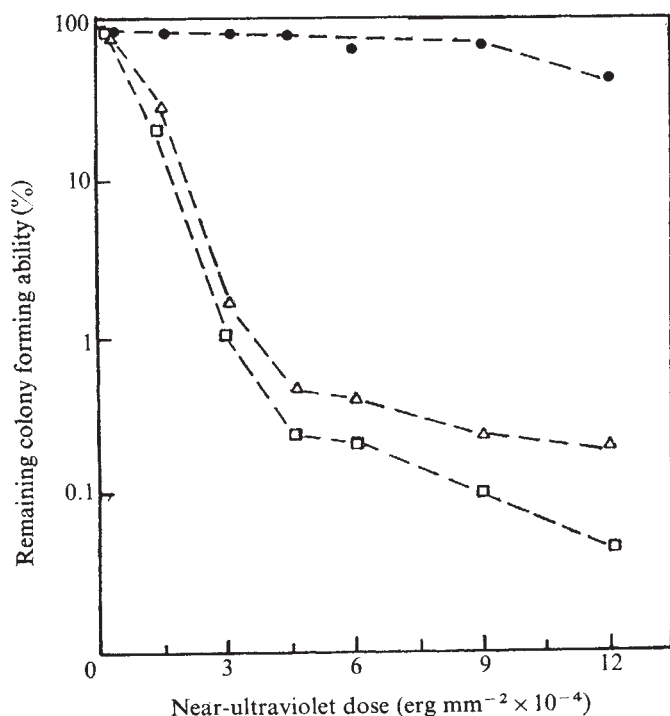


Fig. 2 Percentage of survival of colony-forming ability (●) and plaque forming ability (□) of *E. coli* Hfr H as a function of near-ultraviolet dose with *a*, T₇ phage and *b*, MS-2 phage. Irradiation conditions were as for Table 1. Irradiated cells were infected with T₇ or MS-2 phage at a multiplicity of 0.001. After 8 min adsorption at 37 °C the number of infectious centres was determined by plating with 5 × 10⁶ unirradiated indicator bacteria on L-agar supplemented with 0.1 g l⁻¹ MgSO₄ and 0.2 g l⁻¹ CaCl₂. The total number of plaques was corrected for the number of chloroform resistant unadsorbed phage. In parallel experiments the colony forming ability of identically irradiated but uninfected cells was determined on L-agar.

TAU-bar as a function of the near-ultraviolet dose. Philips HPW 125 W black-light lamps (maximum emission at 365 nm) were used as the near-ultraviolet source. The irradiation apparatus² and the spectral characteristics of the light source¹⁸ have been described previously. Table 1 shows that about two-thirds of the RDP-reductase activity was destroyed by a dose of 3.0×10^4 erg mm⁻² and that there was nearly complete loss of RDP-reductase activity with a dose of 1.5×10^5 erg mm⁻². The activities of β -galactosidase and malate dehydrogenase were not significantly affected in this dose range indicating that the photo-destruction of RDP-reductase activity by near-ultraviolet was a specific effect.

Deoxythymidylate may be synthesised by two independent routes in *E. coli*, either by the *de novo* pathway involving RDP-reductase and thymidylate synthetase or by the salvage pathway utilising thymine and a deoxyribosyl donor²¹. When deprived of thymine, strains lacking thymidylate synthetase undergo lethal unbalanced growth termed thymineless death²². If the photoinactivation of RDP-reductase results in a metabolic state analogous to that occurring in thymineless death, it should be possible to abolish or diminish the lethal effect of near-ultraviolet light by providing the culture with an exogenous deoxyribosyl source. This prediction was tested using *E. coli* TAU-bar, a strain which in addition to lacking thymidylate synthetase is also deficient in deoxyriboaldolase²³. The second mutation results in efficient deoxythymidylate synthesis via the salvage pathway by blocking deoxyribose catabolism^{23,24}. Figure 1 shows the survival curves of *E. coli* TAU-bar irradiated in unsupplemented medium, in medium supplemented with 250 μ g ml⁻¹ deoxyadenosine or 250 μ g ml⁻¹ adenosine. With an initial dose of 4.5×10^4 erg mm⁻² rapid loss of viability occurred and was followed by a slower rate of killing with additional irradiation in unsupplemented medium. The inclusion of deoxyadenosine in the medium prevented the killing of cells up to a dose of 9.0×10^4 erg mm⁻². The survival pattern was similar in the medium supplemented with adenosine as in the unsupplemented medium indicating that the sparing effect was specific for the deoxyribosyl moiety. A reduction of the lethal effect of near-ultraviolet light also occurred with the prototrophic strain *E. coli* B/r thy⁺. After a dose of 4.5×10^4 erg mm⁻² survival was 0.020% in the unsupplemented medium, 0.20% in the medium supplemented with 250 μ g ml⁻¹ deoxyadenosine and 4.2% in the medium supplemented with both 250 μ g ml⁻¹ deoxyadenosine and 20 μ g ml⁻¹ thymine (survival curves not shown).

Another property of cells irradiated by near-ultraviolet light is the loss of their ability to support the replication of DNA phages^{25,26}. *E. coli* cells regain the ability to support the replication of T₁ DNA phage after low doses of near-ultraviolet irradiation when incubated in broth before infection suggesting that a metabolic lesion is involved²⁵. If this lesion is the loss of the RDP-reductase, and if other requisite host functions remain sufficiently intact to produce infectious phage, then it would be expected that the replication of RNA phages would not be suppressed by near-ultraviolet irradiation of the host. This prediction was tested using *E. coli* K-12 Hfr H as the host strain and the DNA phage T₇ and the RNA phage MS-2. Since the replication of the MS-2 genome is carried out by a phage specific RNA-dependent RNA polymerase²⁷ there is no requirement for deoxyribonucleotides. The capacity of irradiated cells to support the replication of T₇ phage was lost exponentially while there was a relatively small decline in the cellular viability up to a dose of 4.8×10^4 erg mm⁻². In contrast, cells which had received doses up to 3.0×10^5 erg mm⁻² retained their ability to support the replication of MS-2 phage while cellular viability was reduced to 0.00025% (Fig. 2).

The data presented here are consistent with the interpre-

tation that the underlying event in the killing of exponentially growing *E. coli* cells by near-ultraviolet light, and in the loss of the ability of irradiated host cells to support the replication of DNA phages, is the photoinactivation of the RDP-reductase complex.

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Leader sequence of 71 nucleotides devoid of G in tobacco mosaic virus RNA

EXHAUSTIVE T₁ ribonuclease digestion of the RNA of tobacco mosaic virus (TMV) liberates an unusually large T₁ oligonucleotide (~72 nucleotides) called Ω (refs 1–4). Analysis of partial T₁ ribonuclease digestion products of uniformly ³²P-labelled TMV RNA shows that Ω is no more than 200 nucleotides from the 5'-end of the chain. We have used polynucleotide kinase to fix a radioactive phosphate to the 5'-end of both Ω and intact TMV RNA (after removal of the pppm⁷G 'cap') and determined the sequence of the first 15–16 nucleotides at the 5' terminus of each by two-dimensional homochromatography of partial P₁ nuclease digests. The results show that Ω begins only one nucleotide away from the 5'-end of the RNA chain, the terminal sequence being m⁷GpppG Ω . . . Consequently, positions 2–72 of the TMV RNA molecule are devoid of G (guanosine residues).

An early attempt to locate Ω in the RNA chain was founded upon the erroneous belief that mild sodium dodecyl sulphate treatment of TMV removes subunits preferentially from the end of the particle containing the 3' OH of the RNA chain⁵. Mandelstam¹ found that Ω was exposed early in the course of such treatment, leading him to conclude that the oligonucleotide was within about 200 nucleotides of the 3' OH end. Recently, however, it has been established that the polarity of sodium dodecyl sulphate uncoating is the opposite of what was previously supposed^{6,7}, implying that Ω is near the 5' rather than the 3' end of the RNA molecule.

As noted above, TMV RNA begins with the sequence m⁷G^{3'} ppp^{3'}G . . . (refs 8,9). The triphosphate bridge which joins the m⁷G cap to the 5' end of the RNA chain proper is resistant to endonucleolytic cleavage, and the RNase-resistant moiety, m⁷GpppGp, can be easily separated from the other products of digestion by ionophoresis at pH 3.5⁸. We have taken advantage of this property to screen a partial T₁ RNase digest of uniformly ³²P-labelled TMV

RNA for fragments coming from the 5'-end. When the partial digest was fractionated on a polyacrylamide gel, seven bands contained significant amounts of the fragments sought (Fig. 1a). Fragments 1-3 were successfully freed of contaminating material by an additional step of gel electrophoresis; fragments 4-7, however, have not yet been purified sufficiently to warrant sequence analysis.

The T1 RNase fingerprint of purified fragment 2 (240 nucleotides) is shown in Fig. 2. The cap structure, m⁷GpppGp, migrates as a diffuse spot to the right of UUG. The corresponding product in the pancreatic RNase fingerprint (not shown) was m⁷GpppGU.

As shown in Fig. 2, fragment 2 contains a large T1 oligonucleotide which we have identified as Ω , in line with the findings of Mandeles¹ (see above). Evidence that the indicated spot is indeed Ω includes the following: (1) treatment of the oligonucleotide with pancreatic RNase gives products in the ratio 4 AAU, 2AAAC, 3 AU, 6 AAC, 6 AC, approximately 13 U and 1-2 C to one G, in substantial agreement with the pancreatic RNase catalogue described for Ω by Garfin and Mandeles⁴; (2) the U₂ RNase catalogues are in reasonable accord except that we find U₅A rather than the U₆A reported by Garfin and Mandeles⁴ and probably U₄A instead of U₅A; and (3) the putative Ω has the same mobility in bidimensional gels as does authentic Ω from a T1 RNase digest of whole TMV RNA, Ω also forms

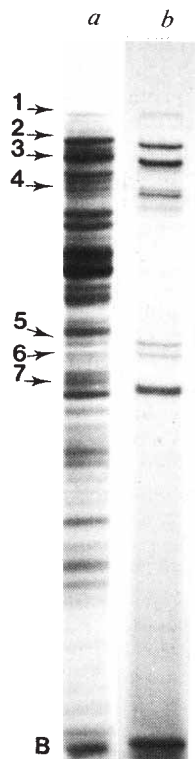


Fig. 1 Polyacrylamide gel electrophoresis of a partial T1 RNase digest of *a*, uniformly ³²P-labelled TMV RNA and *b*, 5'-³²P-labelled TMV RNA. Uniformly labelled TMV RNA was prepared as described by Guillely *et al.*¹⁷; 5'-terminal post-labelling of TMV RNA was carried out after chemical removal of m⁷G (ref. 10) and subsequent phosphatase treatment (0.02 units calf intestinal alkaline phosphatase per 100 µg RNA; incubation for 30 min at 37°C). The buffer was 0.05 M Tris, 0.01 M MgCl₂, pH 7.5. The uncapped RNA (300 µg in a final volume of 0.2 ml) was then incubated for 30 min at 37°C with 2 units polynucleotide kinase and 3 µM γ-³²P-ATP (specific activity: 200 Ci mmol⁻¹). After each step of enzyme treatment in the above procedure the solution was extracted with phenol to inactivate the enzyme, and the integrity of the RNA was verified by electrophoresis in polyacrylamide-Agarose gels. Partial T1 RNase digestion was with 1 unit enzyme per 300 µg RNA in 0.1 M Tris, 0.001 M EDTA, pH 7.5. After 30 min at 0°C the reaction was stopped by phenol extraction and the partial digest was electrophoresed through an 8% polyacrylamide gel¹⁷. Arrows mark the positions of the 5' terminal fragments referred to in the text; B marks the position of the bromophenol blue marker.

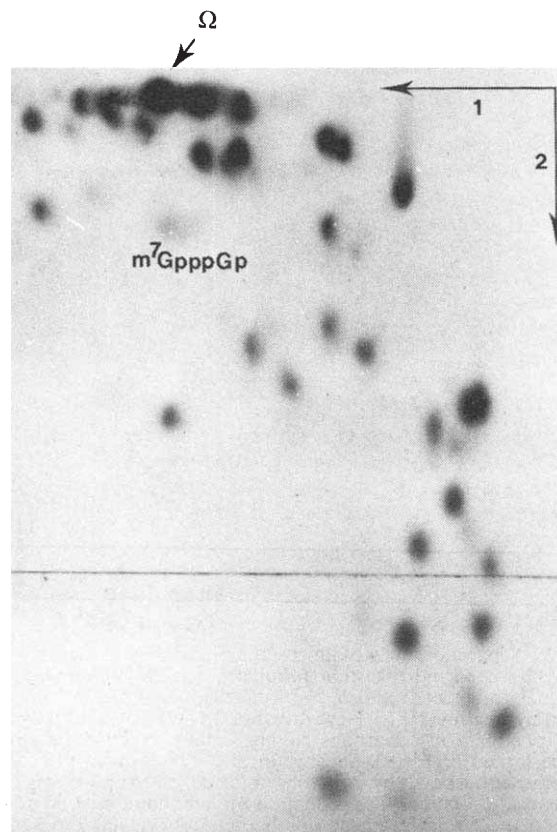


Fig. 2 T1 RNase fingerprint of 5'-terminal fragment 2 of TMV RNA. The first dimension was on a cellulose acetate strip at pH 3.5 and the second dimension on DEAE paper wetted with 7% formic acid.

part of fragment 3 (200 nucleotides) and fragment 1 (approximately 280 nucleotides).

A preliminary investigation of partial T1 RNase digestion products of purified fragment 2 suggested that Ω might lie very near the 5'-end of the sequence. This led us to determine the sequence of the first several nucleotides at the 5'-end of TMV RNA by partial P1 nuclease digestion of 5'-³²P-labelled fragments in the hope that this sequence might overlap that of Ω . But, before a radioactive phosphate can be attached at the 5'-terminus of TMV RNA, the blocking group must be removed. Towards this end, non-radioactive TMV RNA was treated with sodium periodate and with aniline to eliminate the m⁷G (refs 10,11) and the 5'-triphosphate group was removed with alkaline phosphatase. A radioactive phosphate was then fixed to the newly exposed 5' OH group by treatment with polynucleotide kinase prepared from T4-infected *E. coli*¹² in the presence of γ-³²P-ATP.

The 5'-³²P-TMV RNA was partially digested with T1 RNase and the mixture of fragments fractionated by polyacrylamide gel electrophoresis (Fig. 1b). As expected, the seven principal radioactive bands, each of which should represent a fragment from the 5'-end of the TMV RNA chain, correspond closely in mobility with the seven capped fragments detected by screening the partial digest of uniformly labelled RNA for m⁷GpppGp (see Fig. 1a).

The 5'-terminally labelled fragments shown in Fig. 1b were digested with the non-specific endonuclease P1 from *Penicillium citrinum* in mild conditions in order to generate a random population of 5'-labelled partial degradation products¹³. Figure 3a shows an autoradiogram of such a digest analysed by two dimensional homochromatography¹⁴. All seven fragments gave identical patterns. The first dimension of the fractionation was by electrophoresis at pH 3.5 (separation according to charge) and the second dimension by homochromatography (fractionation according to size). Within the limits of resolution in the second dimension, the

18–36 nucleotides. The fragment migrated in the gel with a mobility similar to that of the 30-nucleotide long 2S rRNA from *D. melanogaster*¹⁴.

To determine if the binding is sequence specific, the RNA fragment was eluted from the gel¹⁵ and RNase T1 and pancreatic RNase digests were subjected to fingerprint analysis¹⁶ (Fig. 2). Both fingerprints yielded simple patterns, the number of intense spots agreeing roughly with the expected size of the fragment. This suggested that the binding of IF3 was not random, but sequence specific, although the presence of more than one binding site could not be excluded.

The structures of the main oligonucleotides in the digests are shown in Fig. 3a. Four oligonucleotides in the pancreatic RNase digest correspond to four unique sequences in MS2 RNA (ref. 18), in positions 3,437–3,444 (GAAA-GAGC), 3,453–3,458 (GAAAGC), 3,473–3,479 (GAAA-GGU) and 3,496–3,501 (AGGGAC). This suggests that at least part of the binding site is located in the sequence 3,437–3,501 in the 3'-terminal untranslated region. The remainder of the pancreatic RNase fragments have no unique sequences and therefore cannot be located unambiguously.

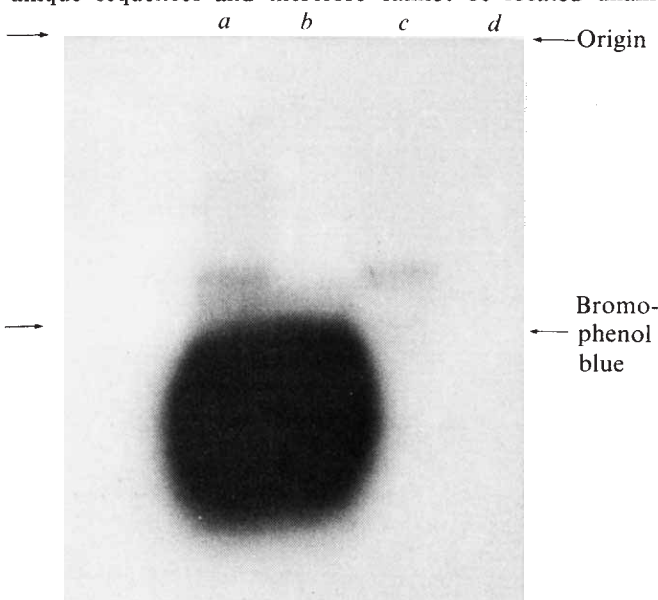


Fig. 1 Electrophoretic analysis of ³²P-MS2 RNA fragment remaining bound to IF3 after digestion with pancreatic RNase. ³²P-MS2 RNA (22.4 µg, prepared as described by Brownlee¹³) was incubated with IF3 (45 µg, prepared according to Lee-Huang *et al.*¹¹ to Step 4, 90% pure) in a total volume of 0.25 ml containing Tris-HCl, pH 7.8 (50 mM), NH₄Cl (50 mM), Mg(OAc)₂ (1 mM), GTP (1 mM) and β-mercaptoethanol (14 mM) for 12 min at 37 °C. Pancreatic RNase (0.5 µg) was added and the incubation continued for a further 30 min at 37 °C. A control incubation was carried out in the same way, but omitting the IF3. The incubations were worked up in one of two ways: (1) Direct phenol extractions (a and b (control)) of the reaction mixture and precipitation with ethanol. The precipitate was collected by centrifugation (30 min at 30,000 r.p.m.) and dried *in vacuo*. (2) Filtration through nitrocellulose (c and d (control)). The reaction mixture was cooled to 0 °C and filtered slowly through nitrocellulose (Millipore, HAWP 02500, 0.45 µm). The filter was washed three times with 10-ml aliquots of buffer (Tris HCl, pH 7.8 (50 mM), NH₄Cl (50 mM) and Mg(OAc)₂ (1 mM)). RNA was extracted from the filter by shaking for 2 min at 70 °C with 2 ml of 1:1 mixture of phenol and a buffer containing Tris-HCl, pH 7.8 (10 mM). The extract was cooled rapidly and the RNA precipitated with ethanol from the aqueous phase. A vertical slab gel (20 × 20 × 0.4 cm) containing 15% acrylamide and 0.375% bisacrylamide in a buffer containing Tris-HOAc, pH 8.2 (40 mM) and EDTA (1 mM) was prepared. The RNA samples were dissolved in 50 µl of this buffer containing 10% sucrose and bromophenol blue, applied to the gel and electrophoresed at 150 V for 16 h at 4 °C. An autoradiograph of the gel was prepared. To prepare large quantities of the RNA fragment, the method described for slot a was used. In a typical experiment, 2 mg of ³²P-MS2 RNA was incubated with IF3 under conditions of maximal binding as determined by nitrocellulose filtration.

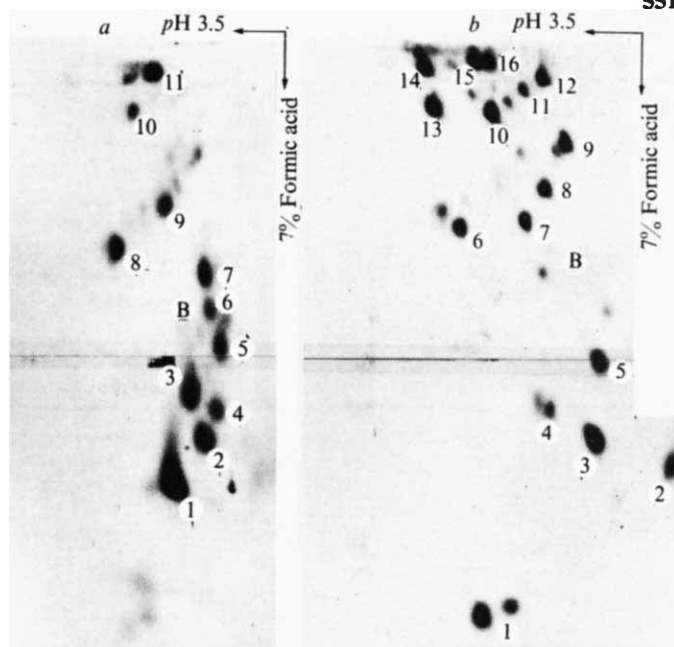


Fig. 2 Autoradiographs of fingerprints of a, RNase T1 and b, pancreatic RNase digests of the protected RNA. B indicates the position of the blue marker. The RNA fragment was prepared as described in Fig. 1, slot a. The band was eluted electrophoretically from the gel¹⁵ and fingerprints of RNase T1 and pancreatic RNase digests prepared¹⁶. The IF3 sample used in this preparation was 80% pure, as determined by SDS gel electrophoresis¹⁷. Fingerprints of RNase T1 and pancreatic RNase digests of the protected RNA prepared in exactly the same way, but using a homogeneous IF3 preparation differed only in the intensities of a few of the spots.

biguously. All except three (AGC, (AG,G)C and (AG,-AG,G)U), do however fit the sequence around the unique nucleotides mentioned. Of the RNase T1 digestion products, none is unique, but all except CCG, CCAG, AACG, UCUCG and (U₃C₃, AC,AU) correspond to the nucleotide sequence in the same region. The RNase T1 product U₃C₃-AC,AU could not be unambiguously identified. It gives one spot on some fingerprints and two on others, but since it lacks a 3'-terminal G residue, it must represent the 3'-terminus of the protected region, which may account for its variability. The 3'-terminal untranslated sequence of MS2 RNA and the location of the digestion products within this region are shown in Fig. 3b. Thus, were the sequence continuous, its minimum length as determined by unique pancreatic RNase products would be 65 nucleotides, that is, twice the length determined by gel electrophoresis.

The pyrimidine-rich sequences 3,466–3,472 and 3,488–3,495 cannot be accounted for in the fingerprints. These regions may be less well protected against pancreatic RNase digestion and/or may suffer some breakdown during isolation of the fragment. But considering the small size of IF3, it seems more probable that these excisions indicate a binding site where different stretches of the RNA are held together in a specific secondary and tertiary structure. This may also account for the presence of the two pancreatic RNase and four RNase T1 products which originate from a more distant part of the MS2 RNA sequence. In this respect, the binding may be similar to the RNA-protein interactions observed between ribosomal RNA and ribosomal proteins where binding does not occur between continuous sequences in either of the participating molecules¹⁹.

So far as is known, the 3'-terminal untranslated region of mRNA has no role in the initiation process and the functional aspects of the binding of IF3 to this part of MS2 RNA remain open to speculation. It is interesting in this respect that S1 protein, which is supposed to participate in the same step as IF3 at the initiation of translation, binds near the 3'-terminus of QB RNA (ref. 10). IF3 may have

a

RNase T ₁ fragments		Pancreatic RNase fragments	
1. G	8. UG	1. U	9. (G,AAAG)C
2. CG	9. UCCG	2. AC	10. GGGC
3. AG	10. CUCG	3. GC	11. (G,G,AG)AC
4. CCG	11. UCUCG	4. AU	12. (G,AG,AAAG)C
5. CACG	12. (U ₃ ,C ₃ ,AC,AU,)	5. AGC	13. GGU
6. CCAG		6. GU	14. GGGU
7. AACG		7. GGC	15. (G,AG,AG)U
8. AAAG		8. (AG,G)C	16. (G,G,AAAG)U

b

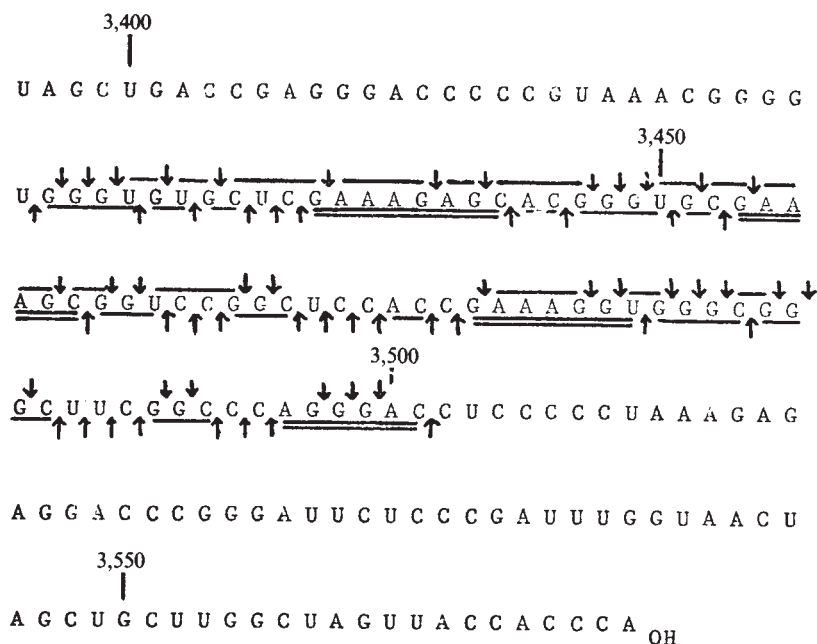


Fig. 3 a, Main oligonucleotides identified in the fingerprints shown in Fig. 2. Secondary analyses were carried out using standard techniques¹⁶. b, The 3'-terminal untranslated sequence of MS2 RNA (ref. 18) and the location of the RNase T₁ and pancreatic RNase digestion products. The RNase T₁ products are shown by a line above the sequence and the pancreatic RNase products by a line below. The four unique pancreatic RNase products are indicated by a double underlining. Arrows indicate nuclease cleavage sites.

multiple binding sites thus stabilising a specific 3-dimensional structure in which the 3' end is linked to some other functional part of the molecule. (Multiple binding sites on mRNA have been found for other proteins^{10,20}). Alternatively, during the course of initiation complex formation the interaction of IF3 with MS2 RNA may be altered by the presence of other protein factors. These possibilities need further investigation. An indication that this binding site might have some relation to the known function of IF3 is the presence of the tract ACCUCC, at position 3,500–3,505, overlapping the binding site. This sequence is homologous to the 3'-terminus of 16S rRNA which anneals to the initiation sites in mRNA and in the neighbourhood of which IF3 is bound to the ribosome^{2,4,5}.

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A histone-like protein from adenovirus chromatin

THE DNA in adenovirus is complexed with two viral specific polypeptides, VII and V, to form the compact core structure. There are 1,070 copies of VII (18,000 molecular weight) and 180 copies of V (45,000 MW) per viral genome of 36,000 base pairs¹. Corden *et al.*² have suggested that the combination of six copies of VII, one copy of V and 200 DNA base pairs form the basic subunit structure of adenovirus chromatin. Such arrangement is also characteristic of the eukaryotic chromatin subunit which is composed of eight histones (two each of H2a, H2b, H3 and H4) and a unit DNA length of approximately 200 base pairs (see ref. 3 for review). The major core protein, VII is highly basic; almost 23 mole per cent of the residues are arginine^{4–8}. A simple calculation reveals that the mass ratio of VII : DNA is 1 : 1 which is similar to the histone : DNA ratio in eukaryotic chromatin. This suggests that polypeptide VII–DNA interaction may be similar to that

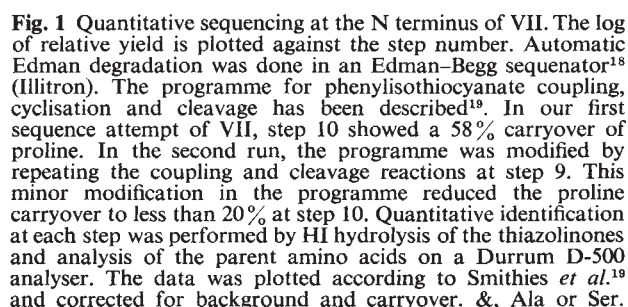


Table I gives the composite sequence data of the N-terminal 40 residues from polypeptide VII. This data is confirmed by two independent quantitative sequence studies (Fig. 1). Our actual sequence data extends to residue 48; however, residues 41-48 have only been sequenced once and are therefore not reported here. It is apparent that the

Figure 2 shows a diagrammatic representation of the charged amino acids in the amino third of polypeptide VII. The asymmetric basic versus non-basic 'domains' as described above are clearly illustrated. In addition, two clusters of basic residues, a triplet of Lys 3-Lys-Arg 5 followed by a longer cluster of His 23-Lys-Arg-Gly-Arg 27 are observed. In between the clusters, His and Arg are segregated at near regular intervals; Arg 11, Arg 13, His 15, and Arg 17 are equidistant from each other while His 8 and Arg 11 are longer by one residue. These non-random distributions of basic amino acids have also been observed in histones^{8,9}, especially in H4, an arginine-rich histone. In H4 (1) about 45% of the first 20 residues are basic, (2) there is a cluster of five consecutive basic residues Lys 16-Arg-His-Arg-Lys 20, and (3) basic residues 3, 5, 8, 12 and 16, are almost equidistant from each other.

The similarity of cationic residue distributions in VII and a highly 'sequence-conserved' histone, H4, immediately raises the possibility that the basic region in VII may also serve as a DNA combining site. The fact that functionally related yet distinctive modifications do occur at the amino end of both DNA-combining basic proteins tends to support this view. As shown in Fig. 2, Ser 1 and Lys 5, 8, 12 and 16 of histone H4 may be modified by phosphorylation¹⁰ and acetylation¹¹. Sung and Dixon¹⁰ have suggested that phosphorylation as well as acetylation of these residues reduces the strong electrostatic binding to DNA at this region of the molecule. This may in turn facilitate the 'proper binding'¹² and assembly of histones into chromatin subunits¹³. It is known that polypeptide VII is synthesised as a precursor molecule P-VII (Pro VII⁹) which is packaged in immature virions^{14,15}. At the final stage of virus maturation, a specific endoprotease¹¹ processes a 20-residue fragment from the amino end of the molecule⁹ to give rise to polypeptide VII. It is likely that this processing event may be related to adenovirus chromatin assembly. We have shown⁹ that this precursor fragment is hydrophobic and may also be acidic (amide status unknown). Thus, the presence of this precursor sequence in Pro-VII may serve to modulate the strong electrostatic binding of the basic 'domain' to DNA. The net result is analogous to that exerted by enzymic phosphorylation and acetylation of histone H4. Subsequent processing event of polypeptide VII enables this region to regain its binding capacity and, thus, allows assembly of adenovirus chromatin.

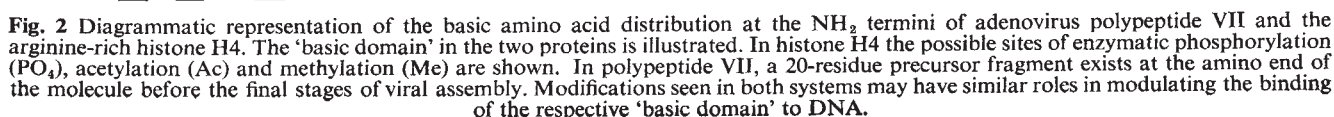


Table 1 Summary of sequence data obtained from the N terminus of VII

Residue no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
HI	&	K	K	R	&	B	Z	H	P	V	R	V	R	G	H	Y	R	&	P	ξ
TLC	A	K	K	+	—	D	E	H	P	V	+	V	+	G	+	Y	+	A	P	W
Sequence	A	K	K	R	S	D	E	H	P	V	R	V	R	G	H	Y	R	A	P	W

Residue no.	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
HI	G	&	H	K	R	G	R	X	G	R	X	X	V	B	B	&	I	B	&	V
TLC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sequence	G	&	H	K	R	G	R	T	G	R	T	T	V	B	B	&	I	B	&	V

The thiazolinones were converted to the phenylthiohydantions and the PTH-amino acids identified by thin layer chromatography²⁰ (TLC). The sequence deduced is given in the last line of the table. &, Ala or Ser; ξ, gly+ala; X, α-aminobutyric acid; —, no PTH-amino acid present; +, not attempted.

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A new theory of olfaction based on dispersion-induced optical activity

OPTICAL activity is physically induced in achiral solutes dissolved in chiral solvents and is measurable as dispersion-induced circular dichroism (DICD)^{1–3}. An intermolecular vibrational coupling mechanism of the induction² has been analysed theoretically⁴ and is here proposed as the primary physical process in olfaction. This new theory of olfaction extends the well-known qualitative vibrational theory^{5–7}, to encompass both chiral^{8,9} and achiral odorous substances^{10,11}. A quantitative aspect of the new theory is supported by a strong correlation of the solvent-induced rotatory power of achiral ketones with their effectiveness as alarm pheromones for the ant species *Iridomyrmex pruinosus*¹¹.

The rotatory inducibilities⁴ of 26 aliphatic ketones were measured by circular dichroism and isotropic absorption spectroscopy in their $n-\pi^*$ transitions as the chiroptical anisotropy factors¹² $g = \Delta\epsilon/\epsilon$, where $\Delta\epsilon$ and ϵ are the dichroic and isotropic molar extinction coefficients respectively. The values of g obtained for solution in (–)-bornyl acetate (BAC) and in (S)-propylene carbonate (PC) (refs 2, 3) are shown in Fig. 1 together with the pheromone activities of the ketones reported by Blum *et al.*¹¹ from laboratory and field trials.

The g values for acetone (point 1) and the homologous normal methylalkyl ketones from C₅ to C₁₂ (3–10) gave line a by the method of averages, line b was similarly obtained for the 3-, and 4-alkanones lacking α-substituents (11, 13, 14, 16, 18), and line c from the point of intersection of a and b and the experimental points for the cyclic ketones (24–26). The bioassay values for the ketones presented a target or star pattern centred on the common point of intersection of a , b , and c at acetone (1) with the natural pheromone, 2-heptanone¹¹, (5), occurring near the centre of the pattern and with pheromone activity decreasing radially. The near coincidence of these two independent, symmetrical, data patterns is compelling evidence of a correlation between olfaction and induced optical activity.

The linear relationships of the g values are most readily explained in terms of helical molecular conformations imposed by vibrational coupling¹³ on the achiral ketones by the chiral solvent and olfactory receptor molecules and will be discussed elsewhere. Such helical structures are, of course, inherent in naturally chiral compounds.

It is evident that DICD and infrared spectrophotometry should provide a quantitative *in vitro* method for evaluating olfactory response to odorous substances and mimics of natural chemical signal compounds.

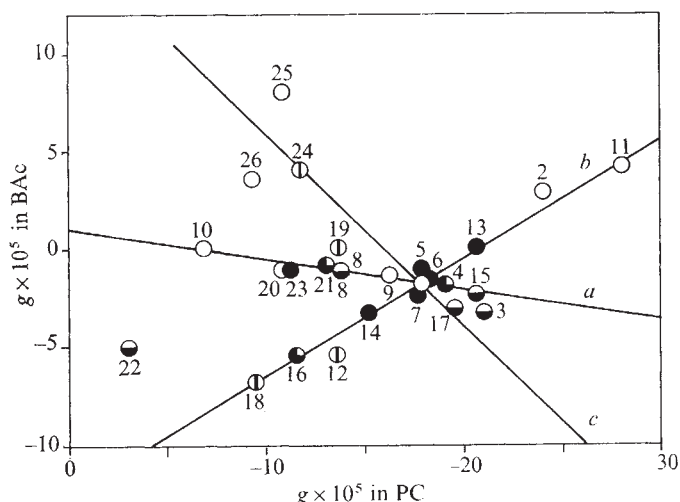


Fig. 1 Correlation of solvent-induced chiroptical anisotropy, g , of achiral ketones in (S)-propylene carbonate (PC) and (–)-bornyl acetate (BAC) with their bioassays as alarm pheromones of the ant *Iridomyrmex pruinosus* (Roger) taken from ref. 11. Pheromone activity increases with filling of the circles from empty at 0 to full at 4. A filled circle also designates the natural pheromone (5). $g = \Delta\epsilon/\epsilon$ was determined from CD and ultraviolet spectra run at $25.0 \pm 0.1^\circ\text{C}$ as described in refs 2 and 3. The ketones are: (1) to (10), the normal methylalkyl ketones from C₃ to C₁₂; (11) 3-pentanone; (12), 4-methyl-2-pentanone; (13), 3-heptanone; (14), 4-heptanone; (15), 5-methyl-2-hexanone; (16), 2-methyl-4-heptanone; (17), 5-nonanone; (18), 2,6-dimethyl-4-heptanone; (19), 3-methyl-2-butanone; (20), 3,3-dimethyl-2-butanone; (21), 5-hexen-2-one; (22), 2,4-dimethyl-3-pentanone; (23), 6-methyl-5-hepten-2-one; (24), cyclopropylmethyl ketone; (25), cyclodecanone; and (26), cyclododecanone.

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Precise location of the crossover region in the λ attachment sequence

A PREREQUISITE for studying the DNA-protein interactions involved in integration and excision of λ is the determination of the DNA sequences of the attachment sites. Useful sequence data can only be obtained if the position of the attachment site is known precisely. To focus on this site

we have used genetically- and physically-defined deletions and substitutions ending in *att* as reference points whose position relative to the sites of action of restriction endonucleases can be determined. In this way we have located the middle of the attachment site to within ± 10 base pairs in a molecule 46,500 base pairs long¹.

The specialised transducing phages λ bio and λ gal carry the right and left prophage end attachment sites respectively, with an adjacent substitution of bacterial DNA and a deletion of part of the opposite end of the prophage (Fig. 1). On hybridisation with normal λ molecules, the heteroduplex molecules formed have a single-stranded region, one end of which corresponds to the junction of bacterial DNA and the attachment site (Fig. 1). One end of one of the double-stranded pieces of DNA that remain after complete digestion of the single-stranded region with the single-strand specific S1 nuclease^{2,3} thus corresponds to this junction. All the available evidence points to this junction being within the attachment sequence and not at the end of it^{9–13}. Thus the region of non-homology in the heteroduplex molecules of λ gal and λ includes the recognition sequences P and B (refs 9, 10), and the region of non-homology in λ bio/ λ heteroduplex molecules includes P' and B' (Fig. 1).

We determined the distance from Bam HI site 3 (ref. 5) to the crossover region by hybridising¹⁴ λ imm⁴³⁴ and λ pgal₈ DNA and digesting sequentially with S1 nuclease and Bam HI. In each of five experiments two small DNA pieces were

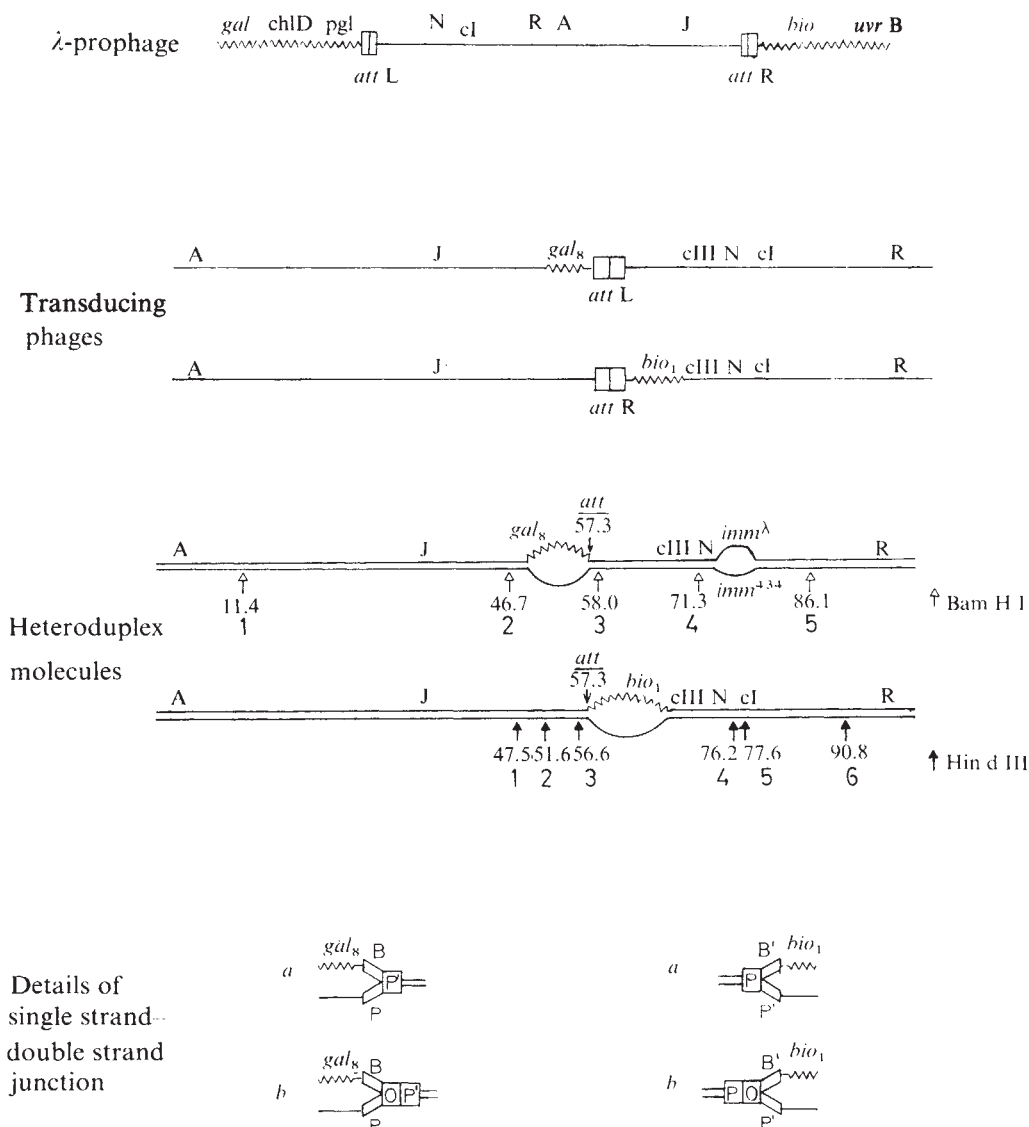


Fig. 1 Structure of λ prophage, λ gal and λ bio transducing phages and heteroduplex molecules between λ and λ pgal₈ (ref. 4) and λ and λ bio₁ (ref. 1), showing the Bam HI (ref. 5) and Hind III (refs 6, 7) cuts. The location of *att* is given at 57.3%, according to Fiandt *et al.*⁸; other authors give it as 57.4%. The detail of the junction of homology and non-homology is drawn, a, without and b, with a common homology region.

detected, one 1,000 base pairs long (probably Bam HI cut 4 to *imm*⁴³⁴, 2.3% λ , 1,070 base pairs), and one 260 base pairs long (Fig. 2a). As a standard for comparison, we used ϕ X174 RFI DNA Hha I fragments, the lengths of which are known from DNA sequencing work¹³. Digestion of *imm*⁴³⁴ or *lpgal*₈ DNA with Bam HI and S1 singly or in combination and digestion of the DNA in the hybridisation mix with either enzyme alone does not give rise to any DNA pieces in this size range. The smaller of the two

pieces can be further positively identified as containing DNA between Bam HI site 3 and *att*, since it can be further digested with Hinf to yield the fragment δ (see below), and a fragment that is 215–220 base pairs long (Fig. 2c). Digestion of λ or *lpgal*₈ DNA with Hind III yields two DNA fragments in the size range 10–1,000 base pairs. One is 620 base pairs long (1.3% λ) and probably represents DNA between Hind III cuts 4 (76.4% λ) and 5 (77.4% λ) (refs 6, 7). The other is only 130 base pairs long,

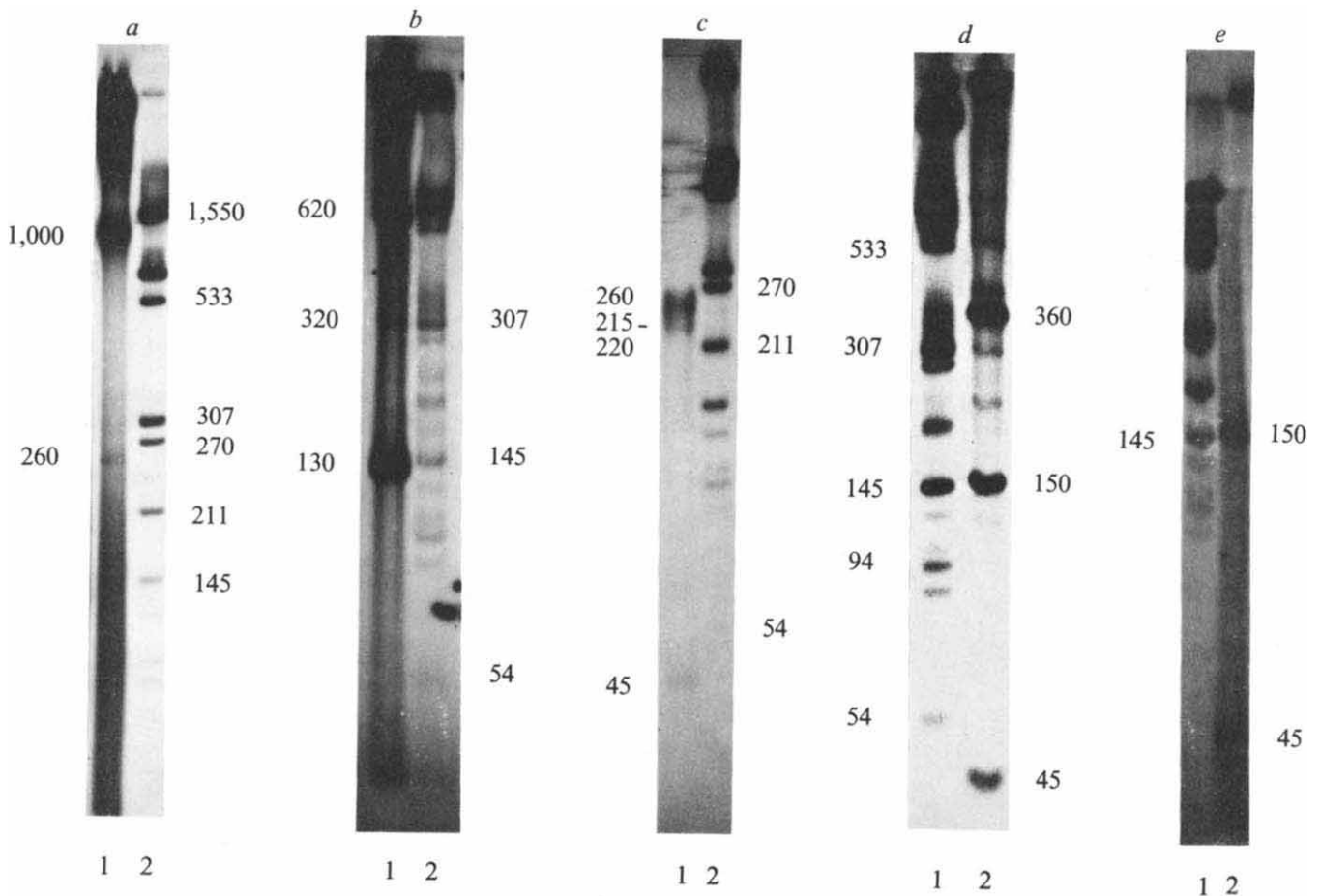


Fig. 2 Autoradiographs of DNA fragments in polyacrylamide gels. *a1*, Phage *λpgal*₈/*imm*⁴³⁴ heteroduplexes treated with S1 and Bam HI; *b1*, phage *λ/lpgal*₈ heteroduplexes treated with S1 and Hind III; *c1*, fragment *att*-Bam HI cut 3 treated with Hinf; *d2*, fragment *α* treated with Hinf; *e2*, fragment *α* end-labelled with ³²P and treated with Hinf. *a2*, *b2*, *c2*, *d1* and *e1*, phage ϕ X174 RFI treated with Hha I. Hybridisation was carried out according to Fanning *et al.*¹⁴. Incubation with S1 nuclease was in 0.03 M Na acetate, pH 4.6, 10⁻⁴ M ZnSO₄, 0.1 M NaCl for 30 min at 22 °C. The DNA was then ethanol precipitated, washed with ethanol, dried and taken up in the buffer for the restriction enzyme digestion. Restriction endonuclease digestion was carried out at 37 °C in the following conditions: Bam HI (purified according to Wilson and Young¹⁶) in 6 mM Tris, pH 7.4, 6 mM MgCl₂, 30 mM NaCl, Hind III (Miles Laboratories and Biolabs Inc.) in 6 mM Tris, pH 7.4, 6 mM MgCl₂, 30 mM NaCl, Hha I (gift of E. Winnacker, and Biolabs Inc.) in 6 mM Tris, pH 7.4, 6 mM MgCl₂, 6 mM 2-mercaptoethanol, 50 mM NaCl, Hinf (gift of A. Waltz) in 10 mM Tris, pH 7.4, 15 mM MgCl₂, 0.6 mM DTT. It was sometimes necessary to separate large fragments from small products of S1 digestion in a 0–20% sucrose gradient run at 41,000 r.p.m. in a Beckman SW41 ti rotor for 14 h at 4 °C before treating with the restriction endonuclease. The source of *α* (Hind III cut 3–Bam HI cut 3) was pMG 1409, a derivative of pSF 2124 (ref. 17) carrying the EcoRI C (ref. 18) fragment. The hybrid plasmid was constructed by *in vitro* ligation of the *λ* EcoRI fragment¹⁸ with RSF 2124 (ref. 17) linearised by EcoRI. The *λ* EcoRI C fragment had been purified using 0.8% Agarose gels and KI equilibrium gradient centrifugation²⁵. The ligation mixture contained 70 μ g of plasmid DNA and 1.5 μ g of *λ* EcoRI C fragment, in a total volume of 1 ml of 50 mM Tris, pH 7.5, 50 mM NaCl, 35 mM MgCl₂, 5 mM DTT, 0.066 mM ATP, with 0.5 U of T4 DNA ligase (Miles). Cells were transformed using the CaCl₂ technique²⁶ and transformants were selected on plates containing ampicillin (100 μ g ml⁻¹). Clones containing the hybrid plasmid were identified by their resistance to ampicillin and their failure to produce colicin EI. Plasmid DNA was purified according to Clewell and Helinski¹⁹ with minor modifications. Digestion of this plasmid DNA with Hind III and Bam HI gives *α* and fragments ~7,000 and 8,300 base pairs long. *α* can be prepurified on a 0–20% sucrose gradient run for 18 h at 41,000 r.p.m. at 4 °C in the Beckman SW41 ti rotor. DNA fragments were labelled *in vitro* by nick translation²⁰ in 50 mM Tris, pH 7.4, 10 mM MgCl₂, 0.1 μ M 2-mercaptoethanol, 5–10 μ Ci of one α -³²P-labelled nucleotide triphosphate (dried to remove ethanol if necessary), the three other deoxynucleotide triphosphates 0.05 mM, with 1–2 units DNA polymerase I (Boehringer, Grade I) for 1 h at room temperature. The reaction was stopped by heating at 70 °C for 10 min. Nick translation usually followed digestion with the restriction enzyme. Fragments were end-labelled by treating with bacterial alkaline phosphatase (Worthington BAPF) for 1 h at 37 °C, extracting once with phenol and once with ether, precipitating and washing with ethanol, taking the dried precipitate up in 20 μ l of a solution containing 1 μ M γ -³²P-ATP (NEN), 50 mM Tris, pH 7.4, 10 mM MgCl₂, 5 mM DTT and 1 mM spermidine, and incubating with 0.5–3 units of polynucleotide kinase (Miles Laboratories, Biolabs Inc.) for 30 min at 37 °C. Samples were subjected to electrophoresis on 8% polyacrylamide slab gels (14 $\times$ 30 $\times$ 0.2 cm). The running buffer was 50 mM Tris borate, pH 8.3, 1 mM EDTA. After running the gel at 15 °C at 200 V for 14 h, the gel was autoradiographed. The numbers adjacent to the bands indicate the numbers of base pairs of the respective fragments.

so there must be a hitherto undetected Hind III cut very close to one of the others. A new band appears when λ and λ pbio₁ DNAs are hybridised and digested sequentially with S1 and Hind III (Fig. 2b). In three separate experiments, the length of this DNA piece was determined as 320 base pairs. This band is not seen when the DNA in the hybridisation mix is digested with either enzyme separately, and represents DNA between Hind III cut 3 and the left end of the bio₁ substitution, since the right end of the bio₁ substitution is 9.7% λ (4,500 base pairs) away from the next Hind III cut^{1,6,7}.

The sum of the lengths of the Bam HI₃-att and Hind III₃-att DNA pieces is 580 base pairs. To obtain an independent estimate of this distance, we isolated the Hind III₃-Bam HI₃ piece (Fig. 2), which we call α . α can be cut by the restriction endonuclease Hinf to yield three pieces, β , γ and δ (Fig. 2d). If the 5' ends of α are labelled using polynucleotide kinase and then digested with Hinf, only γ and δ are labelled (Fig. 2e). A fragment identical in mobility to δ is obtained by Hinf digestion of the Bam HI-att fragment (Fig. 2c). Thus δ is to the right, γ to the left, and β in the middle. The lengths of β , γ and δ as determined by ten separate measurements of their mobilities in gels are 360, 150 and 45 base pairs respectively, giving 555 base pairs for α . No other fragments have been detected, and the radioactivity present in the three bands after nick translation with α -³²P-dGTP is in the ratio 7.4:3.5:1, which is similar to the ratio of the fragment lengths, 8:3.3:1. Endonuclease Hha I also cuts α twice, yielding three fragments that are 470, 65 and 15 base pairs long, their sum being 550 base pairs. MboII also cuts α twice yielding three fragments 380, 90 and 75 base pairs long, their sum being 545 base pairs. These values agree very well with the value obtained from the sizes of the Hinf fragments. We therefore have four independent measurements of the distance between Hind III cut 3 and Bam HI cut 3; 550, 555 and 545 from the Hha I, Hinf and MboII pieces, 580 from the Hind III-S1 plus Bam HI-S1 pieces. The difference is too large to be accounted for by errors of measurement. No significant change in the observed size of the Bam HI₃-att piece was seen on increasing the amount of S1 nuclease twofold or decreasing it fivefold, so digestion of the single-stranded region with S1 was complete. One possibility is that a fragment or fragments have a mobility that does not correspond well to its

real length²¹. A more interesting possibility arises from the fact that if a common region of homology^{22,23} is present in the attachment sequences of phage and host, the ends left by S1 in the attachment region will be at the junction between the common homology region and a recognition sequence (Fig. 1). In this case the ends should overlap by the length of the common homology region, so the discrepancy in the values obtained would indicate a common homology region of 20–25 base pairs (Fig. 3).

The position of the site of recombination is shown in Fig. 3. It is thus possible in this way to determine the physical map position of genetically-defined elements in large DNA molecules to within 20 nucleotides.

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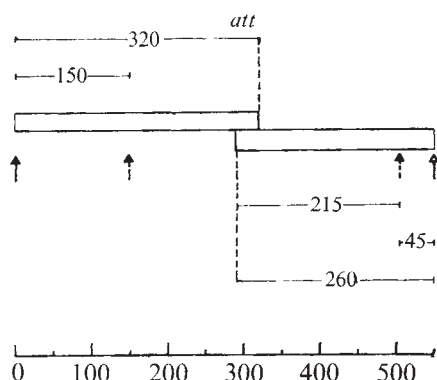
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Erratum

In the article 'Direct demonstration of β -globin mRNA in homozygous Ferrara β_0 -thalassaemia patients', *Nature*, **266**, 231–234, the author sequence should have read S. Ottolenghi, P. Comi, B. Giglioni, R. Williamson, C. Vullo, L. del Senno and F. Conconi. Figure 3 description line 2 should read Nuclear (a) and cytoplasmic (b) . . .

Fig. 3 Map of the attachment site region of λ . The sizes of the Hinf fragments form the basis of the map. Digestion of the Hinf fragments individually with Hha I and MboII places the Hha I cuts 15 and 80 base pairs from the Hind III cut, the MboII cuts 90 base pairs from the Hind III cut and 75 base pairs from the Bam HI cut. Marini and Landy²⁴ have also found a 380 base-pairs long MboII piece containing att. The restriction endonucleases Hpa I, Hpa II, Hae II, Hae III, Hind II, Hind III, Bam HI, Sac III and Pst I do not cut within this DNA segment. Filled arrow, solid tail: Hind III; unfilled arrow, Bam HI; filled arrow, dotted tail: Hinf. Lengths of the fragments are given in base pairs.



matters arising

Function of byssus threads in young postlarval *Mytilus*

STUDY of the settlement of postlarvae of *Mytilus edulis*, which live attached to filamentous algae and hydroids, has shown that they migrate frequently^{2,3}. They detach themselves from their byssus, leave the substratum, are transported again by the water and re-attach on a new substratum. Like Sigurdsson *et al.*¹, we observed long mucous threads on young mussels and compared the drifting at the end of a long thread with the gossamer flight of young spiders, but believed that the aberrant primary byssus thread⁴ was involved chiefly in the settlement of the young. We tested this assumption. We placed a tuft of the alga *Polysiphonia* with many attached mussels in a transparent annular tank (diameter 1 m) in which a constant rotating current was generated. Four small racks with 1.5 m of nylon thread wound around them were placed perpendicular to the current. After 1 d some of the mussels, usually some hundreds, had resettled from the alga on to the threads.

Of the young mussels carried along by the current, occasionally one remained at the spot 2–10 cm downstream a nylon thread, apparently connected by the byssus thread, which had trailed behind and made contact with the substratum. At this anchored stage the elastic mucous thread protruded from between the valves at a point near the apex. Then the animal made swinging movements around this point over an angle of 60°, and in doing so pulled in the byssus thread, again behaving like a spider. Frequently a mussel was able to reach the substratum in this way, but the connection could also be lost. It seemed to be especially difficult for the mussel to grip the substratum with its long motile foot. Sometimes the substratum was abandoned again after short inspection. In such cases time was needed to perform a new anchoring procedure, and this corresponded with the time needed to secrete a new thread.

When nylon threads with diameters in the range 120–1,000 μm were stretched on racks in the sea, the thicker threads caught significantly larger mussels ($P < 0.001$), but such a difference consistently found in the tank was never significant. The average length of the young mussels caught on nylon threads varied in different experi-

ments between 900 and 1,500 μm , the smallest measuring 240 μm (metamorphosis), the largest 2,000 μm or more.

The behaviour of the migrating postlarvae may explain the high resettlement rates found at sea—600 specimens in 5 d—per metre of nylon thread stretched perpendicular to the direction of the current in midwater. In the tank a current of 20 cm s^{-1} was most effective. In the sea the water movement in waves is possibly more important for settlement than are tidal currents.

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Liquid immiscibility in $\text{K}_2\text{O}-\text{FeO}-\text{Al}_2\text{O}_3-\text{SiO}_2$

VISSER and Koster van Groos¹ present a new phase equilibrium diagram for the plane leucite-fayalite- SiO_2 in the quaternary system $\text{K}_2\text{O}-\text{FeO}-\text{Al}_2\text{O}_3-\text{SiO}_2$, in which the field of immiscibility differs significantly from that which I presented earlier², and from that presented by Greig on the binary $\text{FeO}-\text{SiO}_2$ (ref. 3) (Fig. 1). Here I suggest the reason for the discrepancy, as no discussion or explanation is given by Visser and Koster van Groos.

The few details presented on the laboratory procedures used in the new work¹ suffice to indicate that their results should differ somewhat from mine. First, the oxygen fugacity during their work was slightly higher, as they note, because they used molybdenum rather than iron containers. The resulting presence of more Fe^{3+} in the melts would almost certainly expand the field of immiscibility⁴. I am assuming that they were able to overcome the difficult problems of obtaining adequate transfer of oxygen through their evacuated system to achieve PO_2 buffering. Second, the use of a vacuum during the runs in their work might cause loss of

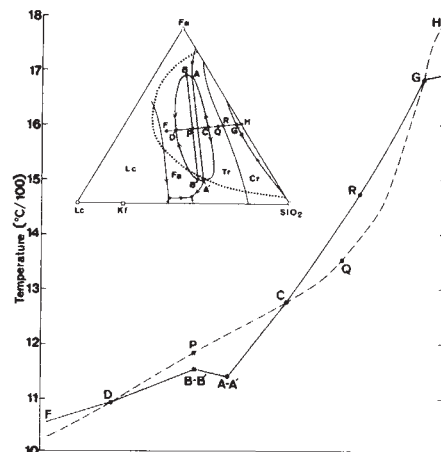


Fig. 1 Phase relations in the system $\text{K}_2\text{O}-\text{FeO}-\text{Al}_2\text{O}_3-\text{SiO}_2$ (inset), showing two fields of stable immiscibility (shaded), projected on the plane leucite (Lc)-fayalite (Fa)- SiO_2 , plotted in wt. %, from the early work of Roedder². The outline of the larger field of immiscibility newly reported by Visser and Koster van Groos¹ is also shown (dotted). Tr, tridymite; Cr, cristobalite; Kf, K-feldspar. The T-X section along the line F-H is based on early work of Roedder², plus the work of Watson¹¹ (point P) and Irvine¹⁰ (point Q). Two immiscible liquids exist in stable equilibrium along D-P-C and G-H.

potassium which would also increase the degree of oxidation of the charges. Third, the molybdenum containers must have put some molybdenum into the charges, either as Mo^{4+} or Mo^{6+} , both of which might be expected to increase the field of immiscibility⁵. I do not believe, however, that these three effects, even though additive, are adequate to explain the major differences found in the extent of the field of immiscibility.

Neither the work of Visser and Koster van Groos, nor my work gives the compositions studied nor the temperatures of the individual runs, but the numbers involved—~500 runs on 26 compositions in my work and >150 runs on an unspecified number of compositions in theirs—would both seem adequate to delineate the phase boundaries. (Although my diagram was presented as “preliminary” at the time, it has since been corroborated by additional data⁶.)

The differences between the two studies can be explained perfectly, however, by the assumption that some of immiscibility found by Visser and Koster van Groos took place during quenching, as the melt passed down through a field of metastable subliquidus immiscibility. The existence of such a field under the liquidus surface for silica in the system as I depicted it is self-evident from the geometry of the two immiscibility fields in a T-X section from the FeO-SiO₂ sideline through points C and D on Fig. 1. Furthermore, experimental evidence has been found for the existence of this metastable field, as well as for a metastable extension out under the very flat fayalite liquidus⁷. This evidence consisted of the presence of minute (~1 µm) globules having a high index, presumably high-FeO melt, in those glasses formed on relatively slow quenching, whereas the same charge quenched more rapidly yielded an optically uniform glass. Unfortunately, neither paper provides details on the quenching procedures. In my work, I quenched by dropping the iron foil envelope (~1×5×5 mm) containing the charge on to mercury while still in pure nitrogen. If the results suggested that phase changes had taken place on quench (for example, the minute globules, or dendritic crystals), faster quench was obtained by attaching platinum weights to the charge in the furnace to cause it to sink in the mercury on dropping⁸. All such minute globules were eliminated by this procedure.

The metastable sub-liquidus immiscibility indicated on Fig. 1 was originally drawn solely on the basis of the thermodynamic necessity of metastable extensions of the experimentally determined stable equilibria. It has been verified experimentally by Irvine¹⁰, who determined the stable and metastable phase relations for composition Q (Fig. 1) at 1,350 °C. The stable assemblage consisted of tridymite crystals and a liquid. Irvine was able to realise metastable immiscibility in this composition, with exsolved globules as much as 7 µm diameter, by superheating to 1,550 °C for 1–2 h, then supercooling for 10–15 min before quenching. The metastable solvus was 'roughly bracketed at 1,350 °C', which agrees well with the extension sketched in Fig. 1. Additional corroboration of the phase relations as sketched in Fig. 1 comes from some runs by Watson¹¹, establishing the 1,180 °C isotherm on the upper surface of the stable immiscibility field. He found this isotherm to intersect the stable two-liquid field at point P on the section (Fig. 1).

Metastable sub-liquidus immiscibility is commonly observed in the field of ceramics, and an extensive literature

exists⁹. Visser and Koster van Groos have used a very sensitive test for such immiscibility (electron scanning microscopy on etched surfaces), and they may even have detected such metastable immiscibility in samples that would seem optically homogeneous. Such metastability would explain many of the features they describe that are otherwise extremely puzzling. (Thus the field of immiscibility as drawn by Visser and Koster van Groos indicates a precipitous drop of 517 °C in the liquidus for silica in the system FeO-SiO₂ by the addition of only 1.35% FeO, and the metastable subliquidus extension of the immiscibility field would have to be even steeper). In view of the above evidence, I cannot accept their diagram as representing stable equilibria. I would suggest that the 'submicroscopic' immiscibility reported by them probably is metastable immiscibility from too slow quenching, and that only their coarser globules (as much as to 3 mm) represent stable immiscibility at furnace temperature.

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VISSER and Koster van Groos¹ have delineated a two-liquid phase field in the system K₂O-FeO-Al₂O₃-SiO₂ which is considerably larger than that reported by Roedder². Furthermore, the extent of the revised two-liquid region on the fayalite-silica join contradicts the phase equilibrium data for the system FeO-Fe₂O₃-SiO₂ (ref. 3). In our opinion the new data probably overestimate the extent of the stable liquid immiscibility field of this system for the following reasons.

(1) The submicroscopic glass-in-glass textures found in charges quenched from between 1,250 and 1,400 °C (ref. 1) are probably not equilibrium associations formed at the run temperature. They probably formed as the melt was quenched through a metastable immiscibility field below the liquidus surface. This explanation would account for the anomalous situation of certain conjugate liquid pairs forming

globules no larger than a few tenths of a micron, whereas macroscopic globules up to 3 mm in diameter form in charges differing only slightly in bulk composition¹. The textures figured¹ are identical in scale and morphology to those produced by quenching silicate melts through sub-liquidus immiscibility domes or by heat-treating supercooled silicate liquids^{4,5}.

(2) Previous studies⁶ show that minor amounts of molybdenum (up to 0.5 weight %) are dissolved by melts held in molybdenum capsules, where the oxygen fugacity is buffered at Fe-FeO by gas mixtures. The technique used by Visser and Koster van Groos¹ depends on the equilibration of melt and molybdenum sample container to buffer the oxygen fugacity of the charge, and is likely to be very favourable to the solution of Mo. Experiments in this laboratory show that the addition of 1 wt % molybdenum to the composition 40 fayalite, 26 leucite, 34 silica raises the upper limit of the two-liquid field by >30 °C. This exceeds the difference in temperature between the fayalite-tridymite cotectic and the leucite rich limit of the miscibility gap¹. Solution of molybdenum from the sample container⁶ would extend the stable two-liquid regions, as observed^{1–3}.

Using ⁸⁰Pt ²⁰Rh wire loop sample containers to minimise iron loss from the charge⁷ and controlled oxygen fugacity at the Fe-FeO buffer, we have confirmed the existence of a low temperature stable immiscibility field isolated from the fayalite-silica join, reported by Roedder². Glass beads formed using the wire loop technique have been analysed and show about a 10% loss of K₂O during an 18-h run; the effect of this loss on the geometry of the immiscible field is minor and where we have analysed the run products can be allowed for on the diagram. In conclusion, the statement that "at least for dry magmas an FeO-rich basalt must yield an immiscible granitic liquid during the later stage of crystallisation"¹ would seem to be premature.

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VISSER AND KOSTER VAN GROOS
REPLY—The data which suggested to us that the liquid immiscibility field was significantly larger¹ than previously determined² were based on two independent phenomena. Runs at high temperatures (1,550 and 1,735 °C), made in molybdenum capsules in an induction furnace, showed extensive development of two immiscible liquids for compositions containing up to 7 wt % $\text{Al}_2\text{O}_3 + \text{K}_2\text{O}$. Spheres up to 1 mm in diameter were formed. The P_{O_2} was bracketed between the Mo–MoO₃ buffer and the Fe–FeO buffer (Fig. 1), and is estimated at 10^{-8} . Because the low-temperature two-liquid field at about 1,250 °C was found with compositions containing 8 wt % $\text{Al}_2\text{O}_3 + \text{K}_2\text{O}$, we thought the two immiscibility fields to be continuous. Therefore we investigated the intermediate temperature range between 1,250 and 1,500 °C. In the runs at these temperatures we found only submicroscopic separation between two liquid phases. We interpreted these as a stable phenomena because quench was relatively fast (5–10 s). Development of submicroscopic separation textures was identical to those at lower temperatures where we found a continuous range from large spheres (2 mm) to submicroscopic textures (Fig. 2a (1)). This suggested some kinetic problem for the phase separation at these temperatures. On the basis of the above we suggested a revision of the liquidus in the system $\text{K}_2\text{O} - \text{FeO} - \text{Al}_2\text{O}_3 - \text{SiO}_2$.

Our recent findings, however, indicate that the major criticism of Freestone and Hamilton³ and Roedder⁴ is substantially correct. Thus, a large part of the immiscibility field as depicted in

our paper¹ is metastable, the stable part is in good agreement with Roedder's data². Before elaborating on this, we shall make some remarks on our experimental procedure. Most of our earlier runs were made by wrapping 250-mg samples in molybdenum foil, and sealing under vacuum in quartz tubing. We selected this technique because the wire loop method will most likely result in serious K_2O losses. Bow *et al.*⁷ report 20% loss (open capsules); O'Hara *et al.*⁵ 15% loss (open capsules); Freestone and Hamilton³ 10% (wire loop); while Naslund (wire loop)⁶ considers the loss insignificant. It is very important to keep the $\text{K}_2\text{O} : \text{Al}_2\text{O}_3$ ratio close to 1, or at least at a constant value, because change in this ratio effects the phase relations and the extent of the immiscibility field.

Several high temperature runs were made in an induction furnace, using molybdenum capsules sealed in platinum capsules. In our current research

we use sealed molybdenum capsules in an argon atmosphere. The gas flow through the furnace is set at the Mo–MoO₃ buffer. While these capsules develop hairline cracks during the run, K_2O losses are minimal. Furthermore, quenching times are reduced to about 0.25 s from the previous 5–10 s. As shown in Fig. 1, the prevailing molybdenum buffer, is Mo–MoO₃, the partial pressure of MoO₃ still very low.

In the experiments, the FeO loss to the sample holder was found to be insignificant; furthermore, the MoO₃ content of the silicate glasses is less than 0.1 wt % (detection limit) in all runs except those made in the induction furnace. Here the MoO₃ content of the glasses was about 0.6 wt %.

With our current method we repeated the high-temperature (1,735°, 1,550 °C) runs. We were unable to reproduce our previous results showing immiscibility textures. Comparison with the previous analytical data show that the $\text{K}_2\text{O} : \text{Al}_2\text{O}_3$ ratio of the earlier runs was very low (0.1) as compared with the new data (0.6–1.1). The MoO₃ content of these glasses is below the detection limit. It seems therefore that the low $\text{K}_2\text{O} : \text{Al}_2\text{O}_3$ ratio, possibly combined with the high MoO₃ content, resulted in the formation of immiscible textures. Results of the larger scale separation were in excellent agreement with our earlier runs. The submicroscopic separation features, however, were not reproduced in most runs owing to a better quenching method.

The submicroscopic phase separation was investigated with an energy dispersion in combination with a scanning electron microscope. Tie-lines of the co-existing liquids are much longer than those determined from the microscopic scale phase separation in the same sample. The lengths of these tie-lines roughly delineate the immiscibility field, as it was shown earlier¹, supporting the revised interpretation that this extend of this field represents the metastable extension of the two immiscibility fields found previously².

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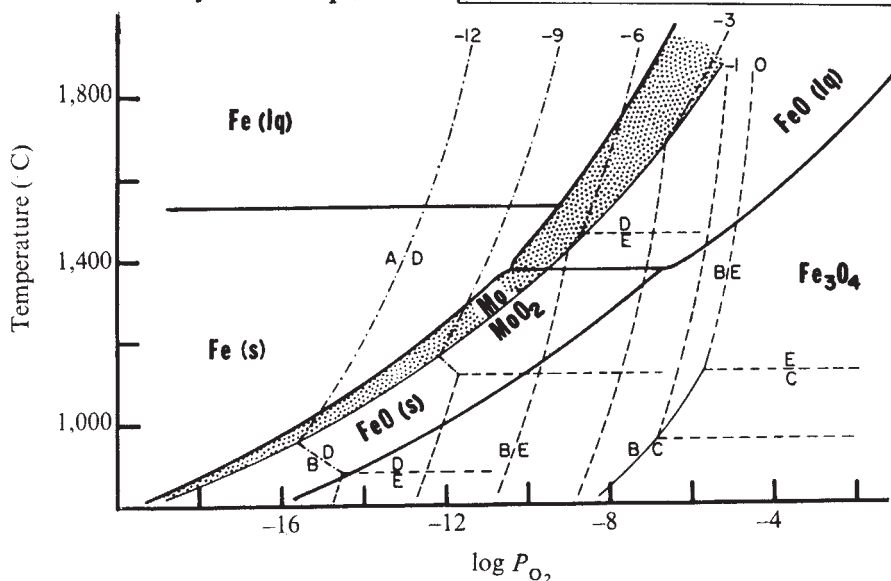


Fig. 1 Systems Fe–O and Mo–O. The heavy line depicts the stability relations of the Fe–O system. The lightly drawn lines depict reactions in the system Mo–O which are independent of the MoO₃ and (MoO₃)₂ vapour pressures, while the dashed lines depend on the (MoO₃)₂ pressure and the dot-dashed lines on the MoO₃ pressure. Runs were made in shaded areas. A, Mo (metal); B, MoO₃; C, MoO₃ (liquid); D, MoO₃ (vapour); E, (MoO₃)₂ (vapour). Data from ref. 7.

Matters Arising

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reviews

Popularising chemistry

A. R. Todd

New Worlds in Chemistry. By Martin Sherwood. Pp. 234. (Faber and Faber: London, 1977.) £6.95.

WITHOUT CHEMISTRY and the great industries based upon it, material civilisation as we know it has no future. The enormous changes which have taken place in the pattern of our daily lives during the past century or so have depended almost entirely on the progress of chemistry—the most central of the sciences whose tentacles reach out through all the others. Without it, the translation of most of the discoveries made in other sciences into practical, usable forms would simply not have occurred; and what is true of the past will be equally true in the future. Despite this, chemistry is undoubtedly less well known and well regarded than, for example, physics or biology by the general public, and it is rarely the subject of popular presentations on radio, television, or in the press. There is no single reason for this state of affairs, but from the many that could be adduced I would choose two as being of major importance.

The first is that the very ubiquity of chemistry and its products makes it a generally accepted part of everyday life to be noticed only when something untoward occurs, whereupon the public reacts with a display of frequently unfounded fear or panic. People, in other words, are aware of chemistry only dimly through its products or applications and are, for the most part, ignorant of or indifferent to the science itself.

The second is that chemistry, however absorbing it may be to its adherents, is inherently difficult to 'popularise'. To be understood, it requires acceptance of certain abstract concepts such as valency, bonding, and three-dimensional orientation of valencies, which have no real counterpart in everyday life. As a result the 'man-in-the-street' is usually unable to appreciate the chemist's ideas of molecular structure and interactions. This inability is not in my view simply due to chemical jargon. Be that as it may, popular expositions of the science have been few in number and successful ones even fewer. The appearance of *New Worlds in Chemistry* is therefore of more than passing interest.

In his new book, Dr Sherwood has done an excellent job of putting across a picture of modern chemistry in action. I found it extremely readable—journalistic here and there, perhaps, but probably none the worse for that. It seems to me that he has been successful because, accepting the ubiquity and maturity of the science, he devotes most

of his attention not to the search for spectacular events but to emphasising and exemplifying the part chemistry has played in a number of important developments in industry, in biology and in medicine, and in efforts to understand the origin of life on our planet.

The book opens with two chapters (47 pages in all) on the "Elements" and "Mechanics" of chemistry. Together they form a praiseworthy attempt to give a comprehensible yet reasonably accurate picture of the basis of chemistry today. I found it both readable and informative, but I was left wondering whether the general reader with no previous knowledge of chemistry would not still be in something of a fog after reading it.

The chapter on "Chemistry in the Beginning" gives a very—and perhaps unduly—full account of work on and theories relating to the development of organic compounds of varying complexity under pre-biotic conditions. Opinions vary about the value of much of the work on pre-biotic synthesis, since it is hardly surprising that organic compounds could be produced in conditions which would not support life as we know it. What would be far more

interesting would be the beginning of organisation—the appearance of coupled reactions under close control which is surely the real beginning of life; but of that we still know little.

The sections on the chemistry of life and death, on industrial chemistry and on the significance of chemistry in a variety of human activities from agriculture to fine art are well done, and the book closes with a chapter on "Chemistry and Tomorrow." In this chapter Dr Sherwood is brief and cautious in forecasting likely areas of advance but he devotes a good deal of space to the well worn question of the relationship between science and government, and the social responsibility of the scientist. These subjects are, of course, controversial but the author is right to point out the dangers inherent in the growing public awareness of the large scale effects which can be produced by modern technology and the need to prevent disillusion with science by the provision of adequate information. Taken as a whole *New Worlds in Chemistry* is a contribution to this end and it can be recommended to the general reader. □

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Vibrations of crystals

Vibration Spectra and Symmetry of Crystals. By H. Poulet and J. P. Mathieu. Translated by A. Simieiev. Pp. xiv+571. (Gordon and Breach: New York, London and Paris, 1976.) £26.

PROFESSOR Mathieu is well known in France as an author of textbooks on the vibrational spectra of molecules and crystals. This book has evolved from one published in 1945. It is a translation of the original French edition of 1970; unfortunately, this is not stated anywhere in the book.

There are ten chapters on theory. They deal with the symmetry and internal structure of crystals, crystal dynamics, the symmetry of vibrations, and the classical and quantum theories of optical phenomena. The last chapter describes applications of the theory to infrared and Raman crystal spectra. There are seven appendices (on group theory and on X-ray and neutron diffraction), and a bibliography giving 185 references. The objective was "to give a systematic survey of the methods used to count the principal vibrations

of crystals, to determine their symmetry, to investigate their interactions with electromagnetic radiation and to establish the selection rules to which they are submitted".

The book has been written in a clear and helpful style, and will be useful to physicists and chemists concerned with the vibrations of crystals. Unfortunately, the translation is not always good—words such as "inequally", and phrases such as "integer multiple" and "allows to reduce their number" are to be found throughout; "et" occurs in the references instead of "and". An old value is used for the velocity of light, and SI units are not always used. There is a reference to Kitaigorodskii's 1961 book rather than to his *Molecular Crystals and Molecules*, published in 1973. There is no discussion of non-linear optical properties such as stimulated Raman scattering and the hyper-Raman effect.

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Linguistic theory

Integrated Theory of Linguistic Ability. By T. G. Bever, J. J. Katz and D. T. Langendoen. Pp. 432. (Harvester: Hassocks, Sussex, UK; Crowell: New York, 1977.) £14.95.

Is linguistics an empirical science like psychology or a theoretical science like logic? Is it, indeed, a science at all? One is bound to wonder about the status of a discipline that has as many schools and schisms as a political movement.

The great revolutionary figure is, of course, Noam Chomsky, the genius of transformational grammar. In 1965, he proposed a grammar, the so-called "standard theory", which formally specifies two different levels of syntactic structure. The surface structure of a sentence segments it into such constituents as noun phrases and verb phrases; its deep structure involves the same sorts of constituents but arranges them in a way that unequivocally identifies the subject and object of the sentence and other such grammatical relations; deep structure is mapped onto surface structure by transformational rules.

Chomsky's syntactic arguments for this grammar were compelling: most of the trouble has arisen over meaning, and the past decade has seen a continuing 'revolution within the revolution'. The standard theory took the deep structure of a sentence as a blueprint for its meaning, and the surface structure of the sentence as a blueprint for its phonological interpretation. But a group of radical linguists has argued that the theory is riddled with internal contradictions: grammar should derive the surface structure of a sentence not from some syntactic intermediary such as deep structure but directly from its meaning.

An alternative and 'revisionary' group, led by Chomsky himself, has progressively developed first the "extended standard theory", which allows both deep and surface structure to contribute to meaning, and then the "revised extended standard theory", which derives meaning solely from surface structure. There are still other alternatives, not to mention the numerous groups of linguists working outside the framework of transformational grammar.

The editors of this book—for it is a book of readings rather than the integrated text that its title page might suggest—are members of the 'revolutionary old guard' who wish to defend the standard theory, which they con-

sider to have been abandoned prematurely. To this end they have pulled together fourteen papers, most of which are by the editors and their collaborators, and most of which have appeared in print before.

The gist of their argument is that many of the phenomena that have seemed to motivate counter-revolutionary developments, in fact, defy linguistic explanation. Rather they are to be explained on the basis of the psychology of speech perception or pragmatic conventions governing conversation. The argument is remarkably persuasive, but its effect on the status of linguistics

is more questionable. Where many theories compete, it is natural to attempt to confront them with a wider set of empirical phenomena. By deliberately diminishing the set of relevant phenomena, the authors might seem to be moving linguistics towards the threshold of the comfortably insulated world of parlour politics.

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3-D imaging techniques

Three Dimensional Imaging Techniques. By T. Okoshi. Pp. 403. (Academic: New York and London, 1976.) \$15.00; £10.65.

INVENTORS have been fascinated by the problem of binocular or three dimensional (3-D) imaging for many years; G. della Porta (1540–1615) is reported to have drawn stereo pairs, but Wheatstone was probably the first to make a stereoscope. Since Wheatstone's time, many instruments and systems for producing 3-D images have been invented, several of them described in this book, but it must be admitted that none has yet proved to be the solution to become popular and make its backers multimillionaires.

In this book, there are chapters on vision (a brief treatment stressing depth perception), lens-sheet 3-D pictures (that is, pictures seen through lenticular screens, as in picture postcards and, about 25 years ago, in advertisements on the London underground), projection displays (the various devices for cine projection of 3-D pictures) and, of course, holography. Then there is a chapter on the information content of the different forms of 3-D imagery, and finally a chapter for unclassifiable systems; some of these are connected with computer-generated images and others, such as Gabor's cine projection screen in the form of a gigantic volume hologram, are unlikely to be realised in the foreseeable future. Notable omissions are the cine techniques popular in the early 1950s in which stereo pairs were projected on a single screen either in different colours, to be viewed through appropriately coloured spectacles, or in orthogonal polarisations, to be viewed through polarising spec-

tacles; there is also no mention of the Land Vectograph, a polarisation system used for producing prints or transparencies to be viewed through polarisers.

This book is at its best in the explanations of the technical details of the systems, and this is as it should be since the subject really depends on inventive developments. It is less impressive on the physical principles; for example, in the chapter on information a table gives the bandwidths required to transmit 3-D television by different methods, and figures of order 10^{13} Hz are given for wide-view holography; however, there are no comments on the prospects for achieving such a bandwidth and there is no indication of how the holographic image might be reconstructed. Much of the discussion in the chapter on information is aimed at television transmission but since this is probably many years ahead it is unlikely that the chapter will be very useful. Thus, the book is really an inventor's compendium or review on the subject and it is very good of its kind. Some of the practical details about lasers and holography are out-of-date, but this is because the book was originally published in 1972 (in Japanese). The present translation is very competently done by the author.

One may ask after reading Okoshi whether there will ever be a really good system for showing 3-D images? Perhaps not, although it is risky to prophesy; however, there is another method not mentioned by Okoshi, but known to and practised by the ancient Greeks, which gives quite good results—sculpture. Perhaps it will find a place among the Alternative Technologies.

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Hydrogen bond

The Hydrogen Bond: Recent Developments in Theory and Experiments. Vol. 1: Theory. Pp. viii+1-390. Vol. 2: Structure and Spectroscopy. Pp. viii+391-888. Vol. 3: Dynamics, Thermodynamics and Special Systems. Pp. viii+889-1,549. Edited by P. Schuster, G. Zundel, C. Sandorfy. (North Holland: Amsterdam, New York and Oxford, 1976.) Dfl. 450; \$173.75.

It has been a privilege and pleasure to review this treatise. The hydrogen bond is ubiquitous, and the growth in its studies phenomenal. When I recall my student days at Leicester, where Louis Hunter and his group made major contributions using classical methods to the study of the hydrogen bond—and at that time it was thought that this type of interaction occurred only with fluorine, oxygen, and nitrogen—we can realise the enormous strides which have been made in the experimental and theoretical approaches to this phenomenon. Matrix isolation techniques, neutron scattering, magnetic resonance spectroscopies, to name just a few, have added enormous depth and breadth to the subject.

We cannot readily avoid coming across hydrogen bonds in our work; whether we invoke them in proton abstraction mechanisms, or demonstrate them in structural studies. Only very recently it helped to rationalise an apparent anomaly we had observed: a precursor postulated in a room temperature reaction required, after isolation as a crystalline solid, an increase of 100 °C in temperature to give the same product. A crystal structure determination provided the answer (Cameron *et al.*, *Z. Naturforsch.*, **31b**, 1421, 1976): it showed the presence of a 12-membered ring hydrogen-bonded dimer, with some of the shortest recorded O—H . . . N, and N—H . . . S bonds.

These three volumes (detailed in the title) increase progressively in length and cover most aspects of the subject. Deliberately excluded are the mainly biological investigations, and also largely those devoted to the excited state and relaxation kinetics.

I personally found the studies of hydrogen-bonded systems by X-ray and neutron diffraction studies particularly fascinating. The two methods are critically compared, neutron scattering giving the longer X—H bond lengths. This technique also reveals that most hydrogen bonds are not strictly linear, and that in bifurcated hydrogen bonds,

the bifurcation is usually uneven.

The topology of the hydrated proton $H^+(H_2O)_n$ ($n=1, 2, 3, 4, 5$, and 6) is discussed. In most cases, at least two different structures could be considered for a given value of n .

Examples of symmetric $H_3O_2^+$ ions (for example, yttrium hydrogen oxalate trihydrate) and asymmetric $H_5O_2^+$ ions (for example, picrylsulphonic acid tetrahydrate) have been observed by diffraction methods. Similar studies by spectroscopic techniques show the important advances made in this field, and suggest that the symmetric diaqua-hydrogen ion $(H_2O.H.OH_2)^+$ is rather more prevalent than the asymmetric mono-aqua-oxonium ion $(H_3O^+.OH_2)$.

Major advances in hydrogen-bonded systems have been made using nuclear magnetic resonance spectroscopy and the use of this technique is beautifully demonstrated for the liquid and the solid state.

Perhaps the last two chapters of this treatise, devoted to liquid water and ice respectively, demonstrate most vividly the diversity and complexity to which hydrogen bonding can give rise.

Although the distinguished contributors number almost forty and come from different nationalities, these volumes are written in excellent English, and overlap between chapters is

minimal. The editors must be congratulated. Most chapters cover the literature up to the period 1973–74, some with appendices and notes added in proof up to 1975, a very creditable achievement in an edited treatise. The volumes are beautifully produced and printing errors are scarce. It is a pity that blurring of pp 1,337–1,352, at least in my copy, slightly mars the overall impression. All volumes contain a subject index (covering all three). Volume 3 has also an author index.

Experimentalists (like myself) however, may well find volume 1 heavy going. It is a pity that individual volumes do not seem to be available.

I have so far only considered the quality of this treatise. Alas, we must also pay attention to its cost. A price of more than £100 will deter individual buyers and many libraries in this country. A crying shame as these volumes with their wide, almost universal, appeal ought to be on all library shelves.

I was instructed and stimulated by reading these volumes; I am saddened by the thought, that not enough scientists may be able to share this experience.

Robert A. Shaw

Robert A. Shaw is Professor of Chemistry at Birkbeck College, University of London, UK.

Immunological recipes

Techniques in Clinical Immunology. Edited by R. A. Thompson. Pp. 241. (Blackwell Scientific: Oxford, 1977.) Paperback £6.25.

BLACKWELL Scientific Publications have over the years accumulated a long list of 'titles' in immunology. The standard they and their authors have maintained is generally high. The books range from accounts of the proceedings of specialist conferences to the practical handbook under consideration here. This latter has eleven chapters on techniques commonly used in clinical immunology. It is parochial in that the kind of immunology under consideration is that practised in British hospitals and research institutes—it makes little or no attempt to be international.

Each chapter is written by a person who is involved on a day to day basis with the methods described. Every chapter starts with a short theoretical introduction to the topic before launching onto the collection of recipes which forms the bulk of the text. I am stuck, without doing the cooking myself, in trying to decide whether the

recipes work. They look alright.

The emphasis is toward the classical formulations of immunology rather than the new cell-mediated brand. There is, for example, no chapter on skin-testing. Little is said throughout the book of the indications for any particular test and only rarely about interpretation of results. Perhaps the most thoughtful chapter is that by Irene Batty on quality control. Few of the previous chapters on such disparate matters as the quantification of immune complexes, agglutination of cells or neutrophil leukocyte function tests (an intriguing choice this for an immunology text), give an indication of the way of handling the information gained. Dr Batty lays out in a clear way the basic necessity of quality control charts and describes the area of activity for which standardisation procedures are possible. Relatively few of the other chapters fall into this category.

It is a useful book carefully compiled which is too expensive for a paperback that might sell well.

A. J. S. Davies

A. J. S. Davies is Professor of Immunology in the University of London, working at the Chester Beatty Research Institute, London, UK.

Climatic reconstruction

Tree Rings and Climate. By H. C. Fritts. Pp. xii+567. (Academic: London and New York, 1977.) £16; \$35.

THIS is the first book to deal comprehensively with the relationships between tree-rings and climate, and their use to reconstruct past climates. By far the greatest volume of research in this field has been carried out in the United States and the majority stems from the Laboratory of Tree-Ring Research in Tucson, Arizona. Although tree-ring research has been carried out in the arid south-western United States and in Europe for over 50 years, it has been the development of a rigorous statistical approach, and the use of the newer multivariate techniques by Fritts and his colleagues at Tucson, that has transformed tree-ring studies from a dating technique to a climatic reconstruction science of world-wide application.

This book is thorough in its approach and assumes little previous knowledge. In particular, the author reviews the biology of tree growth from the molecular level through cellular to the whole tree and presents this account in a clear manner uncluttered with jargon. Some may feel that this aspect has been overplayed, as nearly 200 pages of the book are devoted to aspects of tree biology. Many scientists approaching dendroclimatology do so, however, from a non-biological background and would find the clear accounts in these early chapters most helpful. Following this biological introduction, the climate-growth relationships are examined and a series of models are proposed to explain a variety of observed relationships. There follows a rather brief account of the mechanics of tree-ring dating from sample collection, preparation and measurement to tests of reliability and the statistics used to describe tree-ring series. Included in this section is a mention of the techniques of time-series analysis that have been applied to tree-ring series.

The following chapters, which contain the real meat of the book, deal with the calibration of ring-width with climate and the concept of the response function. In this area, where the mathematical manipulations are difficult, Fritts succeeds in introducing each new concept and provides plenty of examples to show its application. In particular, he justifies and explains the use of eigenvectors of climatic variables in the multiple regression with ring-widths. He works through a number of examples and offers some

biological interpretations. This technique, which circumvents the problems caused by relationships between the variables, has so far been little used by other workers.

Although the response function of a single tree-ring series can be found and attempts made to interpret it in terms of growth models, it is seldom of any use for the reconstruction of past climate as the factors affecting growth are too complex. A factor such as high temperature may be found beneficial at some times of the year, and detrimental at others. Using several study sites, Fritts shows how multivariate techniques often allow valid reconstructions to be made.

The final chapters concentrate on examples of the more recent multivariate approaches—such as, reconstructions of lake levels, river flow, sea temperature, and even fish catches,

which are controlled by sea temperature. The last chapter gives in some detail the recent attempts by Fritts and his colleagues at pressure reconstructions using large numbers of tree-ring stations in the United States. This chapter also looks in detail at the various techniques of verification used to test the validity of the pressure reconstructions.

The book is well produced and clearly printed, and is remarkably free from minor errors. It should be of interest to all climatologists, and to a wide range of biologists, and its readable style compensates for its considerable length and detail. The explanations of multivariate techniques may well be of value to workers in entirely different fields.

Jon R. Pilcher

Jon R. Pilcher is Lecturer in Palaeoecology at Queen's University, Belfast, UK.

Chemical history

Chemists by Profession: The Origins of the Royal Institute of Chemistry. By Colin A. Russell with Noel G. Coley and Gerrylynn K. Roberts. Pp. x+342, 28 plates. (Open University Press, in association with The Royal Institute of Chemistry, 1977.) £9.50.

THE social history of science may seem a vague or obscure enterprise; but the attempt to discover what it felt like to be a chemist or physicist in the past cannot but be interesting, and is valuable because it casts light on that great majority of scientists who do not have a process, a genus or an equation named after them. This history of the Royal Institute of Chemistry, a body which is this year celebrating its centenary, is more than a benign tribute; it is a critical history, with particular emphasis on the gradual emergence of chemistry as a profession as well as a discipline. It is especially strong on the earliest years of the Institute (which did not become Royal until 1944), and indeed on the years before the Institute was founded—we do not get to the birth of the Institute until nearly half way through the book. This emphasis is completely justified by the authors, because unless one had understood what the position of chemists was before 1877 one would not understand the Institute and its problems, many of which have continued well beyond 1877.

The authors trace chemistry back not only to alchemists but also to assayers and pharmacists; and the tension between academics, chiefly preoccupied with the increase of knowledge but also anxious to promote careers for their graduates, and the

practical men, or practising chemists, has remained ever since. At the British Association, Section B devoted to chemistry dates back to 1835; while in 1841 both the Chemical Society—the first in the world, but many years after the Geological and Astronomical Societies—and the Pharmaceutical Society were founded. The Chemical Society was a learned society, run by academics; but with the rise of chemical industry, the invasion by science of industries like brewing, and the passing of laws against pollution, the industrial chemist and the consultant analyst appeared upon the scene in greater numbers, and demanded professional regulation.

On the one hand, there was pressure (especially from the academics) for high standards; and on the other, pressure to include and register all those working in the field of chemistry, many of whom could not pass examinations of honours degree standard. In the event, it was only in the last third of its century that the Institute took these wider responsibilities as its most pressing concern; in the earlier years its professional functions were narrower than they are now—when it has also merged with the learned society from which a hundred years ago it separated. This history does not answer all the questions that one might want to know; it is generous to previous historians, and also to participants in the history, and does not dilate overmuch upon money, patronage or feuds; but it provides a splendid account of its subject, which will interest not only historians but also scientists.

David Knight

David Knight is Senior Lecturer in History of Science at the University of Durham. He has recently published The Nature of Science (Deutsch, 1977).

obituary

A. J. Haagen-Smit

THE FIELD of air pollution lost one of its most vocal and colourful workers when Professor A. J. Haagen-Smit died on 17 March 1977 of cancer in Pasadena, California.

The objective facts are that Arie Jan Haagen-Smit was born in 1900 in Utrecht, Holland, and obtained his B.A., M.A., and Ph.D. from the University of that city, obtaining the last degree in 1928. After obtaining his doctorate, he was successively head assistant in organic chemistry and lecturer until 1936, when he went to the U.S. as a lecturer in biological chemistry at Harvard, spending the 1936–37 academic year there. In 1937 he joined the faculty at the California Institute of Technology as an associate professor, as a part of a conversion of that institution from a local trade school to its present level of excellence, begun under the late Dr Robert Millikan. Haagen-Smit retired at CalTech in 1971 as Professor of Biochemistry. He also served as executive officer of the department of biochemistry and director of the excellent plant environment laboratory there.

His early research interests were in natural products, particularly essential oils, although he made major contributions to the chemistry of plant alkaloids, plant hormones, and the chemistry of microorganisms. Towards the end of World War II, the Los Angeles area began to experience an increased incidence of haze, accompanied by eye irritation and damage to plants. In the late 1940s this had become a sufficiently frequent occurrence to generate public pressure for its abatement, and it was promptly termed "smog" by some supposed analogy to the more venerable phenomenon then characteristic of London. A sizeable effort was made, derived from this analogy, to control emissions of sulphur dioxide in the area. If anything, the situation got worse.

Concerning the events that followed, Haagen-Smit himself subsequently told several stories. I am personally inclined to credit the one he told me when I first met him, in the mid-1950s. Remember that he was particularly concerned with essential oils, and therefore had a very highly trained nose. He said that the current explanation (that it was all a matter of sulphur emissions) did not fit with what he

smelled on a smoggy day, which was much more reminiscent of oxygenated organic compounds. One day he was leafing through some back copies of *Chemical Abstracts*, and chanced upon a report of a Swiss patent from, if memory serves me correctly, the late 1930s. This patent claimed a process for producing mixed oxygenated compounds from hydrocarbons by photolysis in the presence of nitrogen dioxide. He immediately set up a benchtop experiment with some nitrogen dioxide and a bit of 3-methylheptane, and exposed it to a conveniently available ultraviolet light. After a time, he sniffed the resulting brew, and found that the odour closely resembled that of the outdoors air on the same smoggy day. I remember chuckling with him over the question of whether the holder of the Swiss patent should sue the City of Los Angeles for infringement.

Given these facts, it was obvious to Haagen-Smit that the source of Los Angeles pollution was primarily the automobile and secondarily the petroleum refineries that are interspersed throughout the city. (In fact, I have heard it theorised that the initial wartime problem stemmed from the injudicious selection of a site for a plant to manufacture butadiene, a key ingredient in synthetic rubber.) Needless to say, Haagen-Smit was violently attacked. He had shown that ozone was one of the products of the photochemical reaction. Some of the local industries heavily sponsored a local eccentric who purported to have data showing that the ozone was all of natural origin. Industry also funded local research institutions in the construction of rather elaborate laboratory facilities specifically for the purpose of disproving his theory. An elaborate superstructure, the Air Pollution Foundation, was set up to administer these early contracts. Techniques and instruments were devised or adapted for the measurement of a number of the key ingredients in the Los Angeles atmosphere, and these were produced commercially by local scientific instrument makers, particularly by the Beckman Instrument Company. It is probably no exaggeration to say that modern atmospheric chemistry was born from the researches of Haagen-Smit and of those hired by the affected industries to refute him. While there were a few details needing correction, the sole result of the studies was to

support Haagen-Smit's original theory.

Once the issue was settled by the acquisition of further knowledge, Haagen-Smit received numerous appointments, honours, and awards. He served for a time as chairman of the California Air Resources Board and of the U.S. President's Task Force on Air Pollution, as well as serving as a member of numerous other committees. He worked briefly as a consultant for Southern California Edison, and had a signal role in the development of the technique of reducing nitrogen oxide emissions from power plants by two-stage combustion. He was founding editor of the *International Journal of Air Pollution*, but found the work uncongenial and passed the job on to me at the end of the first year. Nevertheless, he served on the Editorial Advisory Board until last year, through two metamorphoses; the journal is now called *Atmospheric Environment*. Honours included membership in the National Academy of Science and receipt of the Los Angeles County Clean Air Award, the Chambers Award of the Air Pollution Control Association, the Hodgkins Medal of the Smithsonian Institution, the B. Y. Morrison Award of the U.S. Department of Agriculture, the Honor Scroll of the American Institute of Chemists, the Fritzsche Award and the Award for Pollution Control of the American Chemical Society, the Cottrell Award of the Research Corporation of America, the John and Alice Tyler Ecology Award of Pepperdine University, and the National Medal of Science. In his native country he was made Laureate of Labour by the Netherland Chemical Society, and he was made Knight of the Order of Orange Nassau. It is almost unnecessary to add that he belonged to a lengthy list of appropriate professional societies.

However, it is not for his honours that his many colleagues throughout the world will remember. "Haagy." Rather we will remember him for his warm humanity, his unflagging humour and his scientific insight. It would be a signal service to the science historians of the future if someone could take the time and the trouble to begin collecting anecdotes of the man. It is perhaps illustrative of his essential humility that I have found it necessary to consult a half dozen biographical sources to compile the above list of

honours; he rated them sufficiently unimportant that he seems to have given no one source the complete list.

A couple of years ago a joke made the rounds in which God's intent to create heaven and earth was completely thwarted by a demand from the Heavenly Environmental Protection Agency that he first file an environmental impact statement. As I fell asleep the night after learning of Haagy's death, that story suddenly returned to me with the thought, "He's got some help; now Creation can proceed!"

James P. Lodge

Donald Reid

DONALD REID, Professor of Epidemiology at the London School of Hygiene and Tropical Medicine, died suddenly of a heart attack on March 26 at the age of 62. His standing as an epidemiologist had few equals, in Britain or in the world.

He was born in a small Scottish fishing village, the son of a Post Office linesman. The family was poor and survived only through his mother's courage and prudent housekeeping. In later life he still preferred a bus to a taxi, and in research he would think long and hard about efficient study design, and how to salvage something if the primary aim were to be frustrated. If "plan A" failed, he always had "plan B" ready.

After qualifying in medicine he entered RAF Bomber Command in 1939, and there laid the foundations of his own career and of psychiatric epidemiology by analysing the relation between stress and neurotic illness. He showed that breakdown correlated highly with current casualty rates in a particular unit, but not at all with total flying hours. After the war he joined Bradford Hill at the London School of Hygiene and Tropical Medicine, and he succeeded him on his retirement in 1961. In a discipline where data are largely observational and often of poor quality he practised and taught scepticism and meticulous accuracy; but where others discounted the research potential of vital statistics, or surveys using crude clinical examination techniques, he showed that even inferior data can be valuable provided that their errors are recognised and quantified.

Most of his work was in relation to respiratory disease, later extending also to cardiovascular problems. Using national sickness-absence data for postal workers he showed the importance for bronchitis of exposure to air pollution: an excess in absence rates in areas with higher fog frequency was specific for bronchitis and for out-

door workers. By similar methods he identified the tuberculosis hazard for workers in bacteriology laboratories and mortuaries. He was a leading contributor to our recognition of the effects on health of cigarette smoking, demonstrating its interactions with air pollution in the causation of bronchitis, and with other coronary risk factors in the occurrence of heart attacks. He was particularly delighted by his recent election as an honorary fellow of the American College of Cardiology, and at the time of his own death he was working on the predictors of death from coronary heart disease.

His reputation stood as high overseas as at home. His international studies of migrants compared their disease rates with those of their native-born contemporaries in the host country and their siblings at home, thus permitting separation of genetic and environmental influences. He had friends in almost every country, and was much in demand as lecturer, chairman and consultant. Many may have thought that his talks and opinions were given impromptu, but in fact they were always meticulously prepared. Similarly his confident and optimistic manner of belied painful inner self-questioning and nervousness.

His colleagues and students will remember him for his humour (often directed against himself), his warmth and unlimited concern with their well-being, and his complete public and private integrity. He leaves his wife and two daughters; and countless friends in many countries.

Geoffrey Rose

M. S. Vallarta

DR MANUEL SANDOVAL VALLARTA died on April 18 in Mexico at the age of 78 after a long and distinguished career. He was particularly well known for his theoretical work on the geomagnetic effects on cosmic rays.

Together with the late Abbé Lemaitre, Vallarta solved to first order the problem of the cosmic ray intensity distribution in the geomagnetic field. This was done in two stages. First, the "allowed" and "forbidden" directions for cosmic ray particles in the geomagnetic dipole were determined, using the newly devised BUSH differential analyser at MIT, by generalising the earlier treatment of Stormer. Secondly, by an elegant application of Liouville's Theorem, he and Lemaitre showed that for an isotropic cosmic ray flux the intensity at the earth in the allowed direction is the full intensity prevailing outside the geomagnetic field whereas that in the forbidden directions is zero.

This work provided the basis of our understanding of a wide range of

cosmic ray geomagnetic effects and led directly to two particularly important results. First, in predicting an East-West asymmetry in the cosmic ray intensity at low geomagnetic latitudes whose sign depended on whether the primary cosmic rays were positively or negatively charged, it made possible the experimental proof that the primary particles are predominantly of positive charge. Secondly, the calculations of geomagnetic cut-off energies as a function of position in the geomagnetic dipole, which were carried out in great detail by Vallarta and his students, permitted for the first time a determination of the form of the cosmic ray energy spectrum in the geomagnetically sensitive region. Subsequently, the use of Liouville's Theorem, pioneered in this context by Lemaitre and Vallarta, has been extended beyond the field of cosmic rays to the general case of charged particle motion in magnetic fields of all kinds, whether they be laboratory accelerators, plasma containment machines or situations where particles are accelerated or trapped in the naturally occurring magnetic fields of a wide variety of astronomical objects.

During the course of his lifetime Vallarta contributed notably to the prestige and advancement of science and learning generally in Mexico. He occupied a number of important posts in education and the administration of science, both in that country and in the wider international scene. In particular, he was *inter alia* and at various times President of the Mexican National Institute of Scientific Investigation, Member of the National Commission for Nuclear Energy, President of the Latin American Council for Cosmic Ray Studies, Member of the International Commission of Weights and Measures, Sub-secretary for Public Education for Mexico City, President of the Institute for Mexican Cultural Relations in North America and Chairman of the Scientific Council of the International Centre for Theoretical Physics in Trieste. He was the recipient of many academic honours in Mexico, Latin America and elsewhere. These included membership of the Pontifical Academy of Sciences, the Colegio Nacional of Mexico, and the American Academy of Arts and Sciences. In 1952 he was appointed a Knight of the French Legion of Honour.

Manuel Vallarta was outstanding in his generation and for many years he has been a familiar figure to successive generations of cosmic ray researchers. His perceptive and penetrating questions will be missed at future meetings and for all who knew him personally his death represents the loss of a good and kindly friend. He is survived by his wife Maria Luisa. H. Elliot

announcements

On the Move

Dr J. P. Caen, after a sabbatical year in the department of Pharmacology, University of Cambridge, UK, back to the Research Unit INSERM 150, Hôpital Lariboisière, 2 rue Ambroise Paré, 75010 Paris, France on 1 July 1977.

Dr N. P. E. Langham, School of Biological Sciences, University Sains Malaysia, Penang, Malaysia, to School of Natural Resources, University of the South Pacific, Laucala Bay, Fiji, on 26 April 1977.

A. Albert, Research Professor in Department of Pharmacological Sciences at State University of New York, Stony Brook, New York 11790, to Emeritus Professor in the Research School of Chemistry, Australian National University, Canberra PO Box 4, ACT 2600, Australia.

Dr S. G. Jennings, formerly in the Department of Physics, Science Laboratories, University of Durham, moved to the Atmospheric Sciences Laboratory, White Sands Missile Range, New Mexico 88001, USA, in November 1976.

Details of changes of department, sabbaticals, where leave will be taken and so on should be sent to On the Move; there is no charge for this service.

Foreign members of the Royal Society elected on 21 April 1977

S. Ebashi, Tokyo University. **I. M. Gelfand**, Moscow University. **E. Hückel**, University of Marburg. **E. Katzir**, Weizmann Institute of Science, Israel.

New Information Services

The Natural Environmental Research Council has set up a Marine Information and Advisory Service (MIAS) which has developed a computerised storage and retrieval system for marine data. For further details contact The Publicity Officer, MIAS, Institute of Oceanographic Sciences, Wormley, Godalming, Surrey, UK.

The Scientific Documentation Centre Ltd has set up weekly Current Awareness Services, handling data on many energy topics. For further information contact Scientific Documentation Centre Ltd, Halbeath House, Dunfermline, Fife, UK.

Person to Person

Wanted, 3-bedroom furnished house or flat for visiting Professor of pathology and wife and 2 teenage children July 1 1977–June 15 1978. Contact G. L. Davis, The Jewish Hospital of St Louis, 216 South Kingshighway Boulevard, St Louis, Missouri 63110 USA.

Exchange 4-bedroom furnished house in Loenen aan de Vecht, 20 min Amsterdam, for 3-4-bedroom house 1 h north or east of New York City 15 September 1977–15 September 1978. Contact G. Mulder, Department of Pharmacology, Free University, Amsterdam, Netherlands.

Marine biologists, aquarists, mariners, naturalists, fishermen. Your observations solicited for scientific work. Author collecting unusual/interesting observations of fresh- and saltwater fish schools, especially observations of spawning. Please give as much detail as possible. Quotations from ships' logs, diaries best. Include location of sighting, date, time of day, kind of fish, behaviour details, observer's name. G. Lorton, Biology Department UVA, Gilmer Hall, Charlottesville, Virginia 22903, USA.

Exchange 4-bedroom detached house in Harpenden, Hertfordshire, 10 min station, 35 min St Pancras, for house in Perth, Western Australia July 1977 to July 1978. Contact G. G. Briggs, Rothamstead Experimental Station, Harpenden, Hertfordshire.

Canadian seeks family accommodation in or near London for one year from August 1977, or exchange house in Montreal. Contact L. Polak, French Department, Birkbeck College, Malet Street, London WC1, or F. McGilly, 4220 West Hill Ave, Montreal 4HB 2S7 PQ Canada.

There will be no charge for this service. Send items (not more than 60 words) to Marcus Dobbs at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

Meetings

13 June–2 July, **Techniques of Developmental Biology**, Rio Piedras, Puerto Rico (G. C. Candelas, Biology Department, University of Puerto Rico, Rio Piedras, Puerto Rico 00931).

20–22 June, **4th International Symposium on Mass Spectrometry in Biochemistry and Medicine**, Riva del Garda, Italy (The Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Mario Negri Institute, Via Eritrea, 62-20157 Milan, Italy).

27 June–1 July, **Workshop on Coastal Pollution Control**, Athens, Greece (WHO, Regional Office for Europe, 8 Scherfigsvej, DK-2100 Copenhagen, Denmark).

28 June–1 July, **Working Group on Constraints in Mental Health Services**, Cork, Ireland (WHO, Regional Office for Europe, 8 Scherfigsvej, DK-2100 Copenhagen, Denmark).

29–30 June, **Strategy and Tactics of Control of Migrant Pests**, London (The Royal Society, 6 Carlton House Terrace, London, UK).

30 June, **Laser Processing of Engineering Materials**, Heathrow Airport (C. D. DesForges, Fulmer Research Institute Ltd, Hollybush Hill, Stoke Poges, Slough, Bucks, UK).

30 June–1 July, **The Evolution of Metalloenzymes, Metalloproteins, and Related Materials**, Brighton (N. Birch, Department of Biochemistry, 9 Hyde Terrace, Leeds, UK).

4–5 July, **Conference on Instrumentation for Ground Vibration and Earthquakes**, Keele (The Institution of Civil Engineers, Great George Street, London SW1).

6–7 July, **Profits, Projects and Solar Energy**, London, UK (J. P. Schlesinger, Hyams & Co., 22 South Molton Street, London W1).

7–8 July, **Symposium on Nutritional Aspects of Food and Agricultural Policies in the UK**, Reading (M. R. Turner, Hon. Programmes Secretary, Department of Physiology and Biochemistry, The University, Southampton).

11–14 July, **People and Computers, Computers and People**, Colchester (M. Brady, Department of Computer Science, University of Essex, Wivenhoe Park, Colchester, Essex).

11–15 July, **Conference on Comprehensive Cancer Control**, Copenhagen (WHO, Regional Office for Europe, 8 Scherfigsvej, Copenhagen, Denmark).

11-15 July, **ECM and ECCM for Digital Communications**, Washington, DC (Continuing Engineering Education, The George Washington University, Washington, DC 20052).

11-15 July, **2nd International Conference on The Organometallic and Coordination of Chemistry of Germanium, Tin and Lead**, Nottingham (J. F. Gibson, The Chemical Society, Burlington House, London W1).

12 July, **Symposium on X-ray Diffraction and Spectrometry**, London (The Meeting's Officer, The Institute of Physics, 47 Belgrave Square, London SW1).

12-14 July, **Recognition Marking of Animals in Research**, London (Marking Conference secretary, RSPCA Headquarters, Causeway, Horsham, Sussex).

14-15 July, **Processing, Structure, Properties and Performance of Polymers**, Nottingham (The Meeting's Officer, The Institute of Physics, 47 Belgrave Square, London SW1).

17-22 July, **3rd International Conference on Cyclic Nucleotides**, New Orleans, Louisiana (W. J. George, Tulane University School of Medicine, New Orleans, Louisiana 70112).

18-22 July, **Symposium on Limitations and Potentials of Biological N₂ Fixation in the Tropics**, Brazilia (J. Dobereiner, EMBRAPA, Km 47, Seropedica, 23460, Rio de Janeiro, Brazil).

21-22 July, **Symposium on Plants and the Atmosphere**, Lancaster (Royal Meteorological Society, James Glaisher House, Grenville Place, Bracknell, Berkshire).

23-26 July, **7th International Congress-Conjugation Reactions in Drug Bio-transformation**, Turku, Finland (A. Aitio, Department of Physiology, Kiinamyllynkatu 10, 20520 Turku 52, Finland).

28 July-26 August, **5th International Course on Techniques in Molecular Biology**, Santiago, Chile (J. A. Allende, Faculty of Medicine, University of Chile, Casilla 6671, Santiago 7, Chile). 2-5 August, **Working Group on Evaluation of School Health Programmes**, Bucharest (WHO, Regional Office for Europe, 8 Scherfigsvej, DK-2100 Copenhagen, Denmark).

5-8 September, **2nd International Symposium on Acetylenes, Allenes and Cumulenes**, Nottingham (J. F. Gibson, The Chemical Society, Burlington House, London W1, UK).

5-9 September, **Course in Fluorescent Microscopy**, Oxford (Royal Microscopical Society, 37/38, St Clements, Oxford, UK).

6-8 September, **International Research Symposium on New Processes of Waste Water Treatment and Recovery**, London, UK (The Conference Secretary, Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

6-10 September, **27th Session of the Regional Committee for Europe**, Munich (WHO, Regional Office for Europe, 8 Scherfigsvej, DK-2100 Copenhagen, Denmark).

13-16 September, **Symposium on Unconventional Photographic Systems**, Oxford (Royal Photographic Society of Great Britain, 14 South Audley Street, London W1, UK).

13-16 September, **4th International Symposium on Nuclear Quadrupole Resonance Spectroscopy**, Osaka, Japan (4th International NQR Symposium, Faculty of Science, Osaka University, Toyonaka 560, Japan).

14-16 September, **3rd National Quantum Electronics Conference**, Southampton (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

16 September, **3rd North West Regional Symposium of the Chemical Society**, Salford (H. Suschitzky, Department of Chemistry and Applied Chemistry, University of Salford, Salford, Lancashire, UK).

18-23 September, **2nd X-ray Powder Diffraction Conference**, Exeter (The Conference Secretary, Phillips Analytical Department, Pye Unicam Ltd, York Street, Cambridge, UK).

19-22 September, **International Conference to Mark the 50th Anniversary of the Discovery of Electron Diffraction**, London, UK (Institute of Physics, 47 Belgrave Square, London SW1, UK).

19-23 September, **2nd International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules**, London, UK (J. W. Garrod, Chelsea College Annexe, 271 King Street, London W6, UK).

19-24 September, **4th Biennial Congress of the International Society for the Study of Behavioural Development**, Pavia, Italy (The Institute of Psychology of the Faculty of Letters and Philosophy of the University, Corso Strada Nuova, 65 27100—Pavia, Italy).

19-24 September, **10th World Energy Conference**, Istanbul (Secretary General of the World Energy Conference, 5 Bury Street, St James's, London SW1, UK).

19-24 September, **8th International Symposium on Earth Tides**, Bonn, FRG (Werke-und Verkehrsamt der Stadt Bonn, Postfach 864, D-5300 Bonn-Bad Godesburg, FRG).

20-21 September, **Population Control by Social Behaviour**, London, UK (Institute of Biology, 41 Queen's Gate, London SW7, UK).

20-22 September, **1977 Autumn Meeting of the Chemical Society**, Aberdeen (The Chemical Society, Burlington House, London W1, UK).

26-28 September, **On-Line Surveillance and Monitoring of Process Plant**, London, UK (Conference Secretariat, 'On-Line Surveillance and Monitoring

of Process Plant', Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

Reports and Publications Other countries—April

Energy, Mines and Resources Canada. Geological Survey of Canada. Bulletin 265: Notes on the Petrology of Nepheline Gneisses Near Mount Copeland, British Columbia. By K. L. Currie. Pp.28. \$4.80. Paper 75-12: The Hay River Formation and Its Relationship to Adjacent Formations, Slave River Map-Area, N.W.T. By G. K. Williams. Pp.17. Paper 75-27: Geology and Engineering Characteristics of Surficial Deposits, Montreal Island and Vicinity, Quebec. By V. K. Prest and J. Hode-Keyser. Pp.27. \$4.80. Paper 76-23: Terrain Characterization and Evaluation — an Example from Eastern Melville Island. By D. M. Barnett, S. A. Edlund and L. A. Dredge. Pp.18 \$3.60. Paper 76-25: VLF Mapping of Geological Structure. By W. M. Telford, W. F. King and A. Becker. Pp.13. \$3. (Ottawa: Geological Survey of Canada, 1976 and 1977.) [124]

Geomagnetism (Solid Earth) Data Services and Publications. Pp.iii+14. (US Department of Commerce: National Oceanic and Atmospheric Administration.) (Boulder, Colorado: National Geophysical and Solar-Terrestrial Data Center, 1977.) [134]

Der Beruf des Geologen in der Gegenwart Von Walter Nabholz. (Bern: Rektoratsreden.) Pp.20. (Bern: Verlag Paul Haupt, 1976.) Fr./DM 4. [144]

Laboratory Evaluations of Compounds as Repellents to Cockroaches, 1953-1974. By O. F. Bodenstein. (Production Research Report No. 164). Pp.28. (Hyattsville, Md.: US Department of Agriculture, Agricultural Research Service, 1976.) [154]

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